Seed viability and *in vitro* culture of *Afrocarpus gracilior* and germination studies on *Ekebergia capensis* and *Pygeum africanum*

by

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DEDICATION

This thesis is dedicated to my mother, Hababo Kalacha and my father, Balcha Mojo. It was their long lasting wishes to see my success in my future endeavours.

ABSTRACT

The conditions which prolong viability and the low germination rate of seeds of *Afrocarpus gracilior* were studied. Different seed lots were stored under various moisture content and temperature combinations. The two seed lots tested had different initial viabilities, probably due to different pre-storage treatments, but generally similar storage requirements to maintain viability at a reasonable level. Tetrazolium tests gave consistently higher viability estimates than germination tests but were well correlated with the latter. Intact seeds were found to have a very low germination rate. However, removal of the seed coat and germination at 30 °C resulted in germination rates of up to 80% after 30 days. The seed coat was found to contain a very powerful inhibitor of germination of *Triticum* seed.

Studies were made on the germination of seeds of *Ekebergia capensis* and *Pygeum africanum*. Intact seeds of *E. capensis* germinated at about 52% under optimum conditions and slight damaging of the seed coat halved the germination rate. Intact seeds of *P. africanum* germinated at about 20% under optimum conditions and slight damaging of the seed coat doubled the germination rate.

Shoots of *A. gracilior* were found to grow well *in vitro* on hormone free medium but did not produce axillary buds during normal growth and development. However, it was found that decapitation resulted in the formation of axillary buds at or near the top of the shoots which subsequently developed into shoots. Treatment of intact or decapitated shoots with benzyladenine resulted in the initiation of numerous buds but it proved impossible to extend them into shoots. Extending shoots rooted spontaneously on hormone free medium and were also rooted *ex vitro* in compost under non-sterile conditions and the rooted plants were weaned to greenhouse conditions.

Excised zygotic embryos of *A. gracilior* were subjected to a wide range of temperature, growth media and plant growth regulator treatments *in vitro*. Callus was frequently observed and proembryos occasionally but only one cell line matured to form somatic embryos.

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ABBREVIATIONS

ABA	Abscisic acid
BA	Benzyladenine
BN	Benzyladenine + Naphthalene acetic acid
DMSO	Dimethylsulfoxide
GA	Gibberellic acid
HCl	Hydrochloric acid
HFSE	High Frequency Somatic Embryogenesis
IAA	Indole acetic acid
IBA	Indole butyric acid
IEDC	Induced Embryonic Determined Cells
KIN	Kinetin
KNO_3	Potassium nitrate
LM	Litvay Medium
MC	Moisture content
MS	Murashige and Skoog (1962)
MSG	Murashige and Skoog with glutamine
NA/NAA	Naphthalene acetic acid
NaoH	Sodium hydroxide
PEDC	Pre-Embryonic Determined Cells
SH	Schenk and Hildebrandt
T	Temperature
TZ	Tetrazolium Chloride
WD	Withdrawal
WPM	Woody Plant Medium
WPMG	Woody Plant Medium with glutamine
2,4-D	2,4-dichlorophenoxyacetic acid

CHAPTER 1

Introduction and Literature Review

1. Introduction

1.1 Background

A natural forest is part of the national heritage of a country. It is a source of food and energy as well as providing shelter to a considerable diversity of wild animals.

According to FAO (1995) closed forests covered slightly over 3,400 million ha or 27% of the world's land area, 18% of Africa. The area of forest remained more or less unchanged over the decade 1980-1990 in developed regions, but in the developing countries deforestation (0.81%) exceeded plantation (0.16%). In the Sudano-Sahelian region, the pressure on forest formation through uncontrolled clearing and fuelwood harvesting is so excessive that it can be considered much higher than natural regeneration capacity (Flandez and Quedraogo, 1994), and in Ethiopia, forest resources have been depleted more rapidly than the African average.

There is a considerable body of evidence to suggest that the loss of tropical forests is leading to unprecedented loss of biological diversity (Whitmore and Sayer, 1992). Wild animals have an important role in forest ecology. For example, insects are involved in pollination while many species of mammals and birds possibly 'scarify' seed coats while acting on pulps for food so that germination is made easier. In tropical countries at least 70% of the resident vertebrate species are commonly reported to be dependent upon closed forests (Johns, 1992).

Woody perennials can have a significant potential soil ameliorating effect. For example, a 1-2% increase in soil organic matter in the range of 4-6% can result in a more than twofold increase in base content, reaching values in the range recommended for agriculture (Montagnini and Sancho, 1990). In Ethiopia, the exotic *Eucalyptus* has limited value for soil improvement because the litter is poorly incorporated and lack of ground vegetation increases the risk of soil erosion, in

contrast to native genera such as *Ekebergia, Erythrina* and *Cordia*. Once these indigenous species are destroyed it is very difficult to regenerate them: Gradwohl and Greenberg (1988) noted the reasons why tropical forests do not reclaim land which has been completely cleared as follows:

1. Seeds of tropical forests are very often spread by animals and the chance of these animals living near the clear areas by adapting to drastic habitat changes is minimal.
2. The degree to which seeds can remain viable in tropical soils in the presence of insect and fungal attack varies greatly between species.
3. Human-generated clearings are very often too stressful for seeds to survive.

Sometimes the primary vegetation may cease producing potential seeds, and today, problems of this type are being solved through clonal propagation. Longman and Jenik (1987) reported that sometimes seeds of primary forest trees are no longer available for natural regeneration, and so the flora and structure of a secondary forest may be very different from that in primary vegetation. Forest production is difficult without the availability of seeds, and a lot of development programmes are hampered because of this problem. One of the major limiting factors to afforestation and reforestation is the lack of adequate seed sources (Ponnuswamy *et al.*, 1991). In developing the technology for storage and treatment of seeds it may not be appropriate to extrapolate temperate experience to tropical species, or experience in the dry tropics to rain forest species. Each case must be assessed on its individual properties, and in relation to local requirement, although the principles are the same (FAO, 1985). Seed testing and handling facilities in tropical regions are limited and hence it is difficult to maintain seed quality. The problems of seed germination testing in the tropics are particularly acute because of variations in available controlled environment facilities, inadequate sampling procedure and substrata phytotoxicity, for example.

1.2 Causes of forest destruction

There are many factors that influence the destruction of forests, such as the history of logging, land clearing and urban development, fire, windstorms, insect and disease epidemics and other happenings that affect the life and growth of the trees (World Resources Institute, 1990).

The growth of human populations is one of the major factors. Kimmins (1987) reported that, as human populations grow, more timber is used and more forest cleared for agriculture, and this leads to further increases in the population and even more destruction of the natural forest. The reasons for deforestation in the tropics are complex (Tacon and Harley, 1990) but it is due, partially, to the exploitation and export of timber to gain foreign currency and in great measure to the use of wood for energy (about 80% is burnt for fuel). Clearance to meet the demands of agriculture is exacerbated by the rise in population: forests are also cleared for construction purposes, and to provide material for farm implements. Reasons for deforestation, and modes of deforestation, vary widely between continents. In Brazil, the single most important cause of deforestation is the development of cattle ranching. Elsewhere, for instance in S.E. Asia, it is the need for export-quality timber which is readily available by selective logging of the Dipterocarp forests. In Africa, the main reasons are conversion of forest to agricultural land, wood demand for fuelwood, construction, farm implements and other products, large scale logging operations, inappropriate land use policy and population growth.

1.3 Consequences of forest destruction

There is a general acceptance that overall forests are being cleared at a faster rate than they are being replanted (Thorpe *et al.*, 1991). The specific case of the Ethiopian forests fits into this general condition. Although afforestation activities have been undertaken by the State Forest Services and many Non-Governmental Organisations, reforestation is far exceeded by the loss of forest area. Natural calamities like drought and flood combined with forest destruction have already

claimed the lives of people in many places. The destruction of woody vegetation which followed the tragic drought of 1968-73 in the Sahel, which repeated itself in the mid-80s in many low rainfall areas, is a typical example of the consequence of crossing the threshold of resource tolerance under mounting population pressure (FAO, 1989).

Highland forests may be valuable to lowland residents as sources of water, but the lowlands can suffer from flooding if the forests are completely cut. This is what is happening in Ethiopia; the country has chains of central plateaux from which forests have been totally removed and as a result soil erosion is becoming an acute problem. Power dams have been broken because of excessive run-off water due to the clearing of vegetation from upper catchments. In most tropical countries there are areas of upland cloud forest and rain forest which ought to be permanently reserved because any disturbance can bring about erosion, flooding or landslides on the lower slopes and in the valleys, sometimes of a magnitude to sweep away or bury whole villages (Westoby, 1989).

Clearfelling of the largest trees can cause the loss of endemic plants and animals for good and the selective removal of good quality timber trees for sawmills has resulted in a reduction in quality of the remaining forests. In the felling process larger trees damage the smaller ones, leaving a mass of fallen debris to smother any advance regeneration and go up in smoke at the next fire season. In Bale, south-east Ethiopia, it has been observed that some local individuals have uprooted the saplings of regenerating trees like *Warburgia ugandensis* and *Afrocarpus gracilior* after the timber enterprises felled larger trees on their coffee plantations to avoid any future felling of trees which might destroy their coffee crops.

Resource protection and saving of endangered and highly valuable species should be planned without further delay. According to Negussie *et al.* (1991), any uncontrolled felling of forests by commercial enterprises should be stopped and, moreover, there should be a sound programme to replace the cleared forest. The expansion of coffee and tea plantations and state farms in Ethiopia has taxed the existence of indigenous forests and unless such destruction is stopped, many of the endemic species will be at risk of extinction. There has to be a sustainable land use

alternative to retention of the land under virgin forest rather than ruthless destruction. Compensatory forest plantations could be an alternative source of timber, construction and fuelwood which would reduce the pressure of further exploitation on indigenous forest resources.

1.4 Significance of the study

Much has been said about the cause and effect of forest destruction in relation to the dismal history of the Ethiopian forests whose area has alarmingly fallen from 40% to less than between 4 and 13% in about half a century. It is therefore high time to undertake studies on how to conserve and propagate forest trees.

One possible method for the conservation of tree species is by storing plant propagules, mainly seeds, over a long period of time. Storage conditions and mass propagation methods have therefore to be optimised to prolong the viability of seeds.

The combination of work on seed storage, seed germination and tissue culture is very important since it approaches the problem from several angles. Unless efforts are made to intensify gene conservation, propagation and wise management, serious depletion of the forest will result (Wang, 1988). Spears (1981) emphasised the protection of ecosystem as follows: unless the present trend is reversed the bulk of the tropical forests could well disappear by the middle of the next century. Techniques to handle recalcitrant seeds (i.e. those which cannot withstand desiccation below a certain level) should be investigated: these are vulnerable to extinction because of unwise logging and excessive land clearance and since the forest floor does not hold a reservoir of recalcitrant seeds (Nkang, 1988), and the development of suitable storage techniques and improved propagation methods is therefore essential.

Seed storage plays an important role in provenance trials for tree improvement programmes at an international level (Emmanuel and Dharmaswamy, 1991) since such programmes need a reliable long term storage system.

1.5 Seed species and specific work

1.5.1 Seed storage of *Afrocarpus gracilior* (Pilger) C.N. Page

The Tree Seed Section at the Forestry Research Centre, Addis Ababa, has standardised the storage conditions for some desiccation-tolerant species. However, further investigation is required to optimise the storage conditions for less orthodox, and recalcitrant, seed species to prolong their viability. Without this there could be future scarcities of seeds, especially of recalcitrant species, for plantation programmes. In the short run it is neither economically nor technically possible for the Forestry Research Centre to investigate the optimum storage and germination conditions for every seed species under its management. However, it is possible to categorise seeds with related characters into groups for assessment purposes. *Afrocarpus gracilior* was chosen for this study because its timber is in high demand, since existing stands are being cleared at an increasing rate, and it was considered representative of a species group requiring urgent research into storage and germination.

1.5.2 Germination studies on *Pygeum africanum* Hook and *Ekebergia capensis* Sparrm

Germination studies for indigenous tree species like *Afrocarpus gracilior* and *Olea africana* have been carried out by the Plant Physiology Section at Addis Ababa University, and by the Forestry Research Centre in Ethiopia. In these projects, some encouraging results have been achieved over the past few years.

However, germination problems of species like *Pygeum africanum* and *Ekebergia capensis* have not yet been tackled and it is not known whether these species are orthodox, recalcitrant or intermediate. Their very low germinability should therefore be investigated to see if this is due to desiccation or to the presence of germination-inhibiting factors which cause seed dormancy.

The seed coats of *Pygeum africanum* and *Ekebergia capensis* are likely to be a physical barrier to water and gases, although softer than *Afrocarpus gracilior* and *Olea africana*, for example. In *Ekebergia* the seed coat is weak at the micropylar

region leading to ingress of moisture and consequent microbial attack during the germination process, and even in storage.

1.5.3 Micropropagation of *Afrocarpus gracilior*

Micropropagation of plants is a modern biotechnology whereby cells, tissues or organs are manipulated to produce a large number of plants of improved quality. By using this technology it seems likely that the limitations of seedlings will be overcome, with genetic gain achieved in a relatively short period of time compared with the conventional tree improvement programmes. Regeneration problems of difficult-to-germinate seed species could be solved by tissue culture techniques and *Afrocarpus gracilior* will also be considered for this work.

2. Literature Review

In this section the maintenance of viability, germination and micropropagation are considered.

2.1 Viability and Storage

Moisture content and temperature are the main factors in determining the fate of seeds in storage. Possible combinations of these factors have been investigated through research work to find out the optimum storage conditions for seed species. In many cases it is generally true that the lower the temperature and the lower the moisture content the longer is the viability of the seeds. However, this is not always the case as the larger-seeded temperate hardwoods (e.g. *Fagus* and *Quercus*) and many recalcitrant species of warmer climates (e.g. coffee and citrus) need a relatively high moisture content for maximum retention of viability (Bradbeer, 1992). According to Chin (1988) the most important factor which determines the life and death of seeds is moisture content.

Orthodox species are those whose seeds resist desiccation and are commonly stored after being dried to a moisture content of 5 ± 1% at -18 °C (Roberts and Ellis, 1984); 5-7% at -18 °C (Berjak *et al.*, 1984; Nkang and Chandler, 1986); 6-7% at between +5 and -20 °C (Chin, 1988) and 1-5% at -18 °C (Farrant *et al.*, 1988). *Araucaria columnaris* seeds, which are orthodox but less so than other orthodox species, can be stored after the moisture content is reduced to 12% at 0-5 °C in the short-term and to 7% at -18 °C or lower in the long-term, although there is some loss in viability (Tompsett, 1984).

By comparison, the seeds of many recalcitrant species quickly lose viability when their moisture contents fall below some critical level (Akoroda, 1986). Recalcitrant seeds are predominantly from tropical and subtropical species with large seeds (Côme, 1983; Farrant *et al.*, 1988), and are short-lived under all conditions. When loss of viability is more rapid than usual under ambient conditions, it is likely (though not certain) that the seeds are recalcitrant (Roberts and Ellis, 1984). According to Farrant *et al.* (1988), recalcitrant seeds are of three categories:

1. Less recalcitrant - those which have the ability to withstand relatively more water loss, e.g. *Araucaria hansteinii*;
2. Moderately recalcitrant - those which tolerate moderate loss of water and show relatively slow rate of germination, e.g. *Hevea brasiliensis*; and
3. Highly recalcitrant - those which tolerate very little water loss and begin germination immediately on being shed, even in the absence of additional water e.g. *Syzygium* species.

The drying method of recalcitrant seeds needs care since the acceptable range of moisture content differs between species. For example, Fu *et al.* (1990) noted that the initial moisture contents of three recalcitrant species seeds (*Mangifera indica, Litchi chinensis* and *Euphorbia longan*) were 70-75%, 45% and 38% respectively and that each had a different threshold moisture content below which damage could occur. To maintain their viability from several days to several months or more it was necessary to keep the seed moisture contents above those safe lines. Several workers claim that many recalcitrant seeds can be dehydrated rapidly within an acceptable range of moisture content and still retain viability. Berjak *et al.* (1990), for example pointed out that rapid dehydration of recalcitrant seeds allows viability retention to a lower moisture content than does slow desiccation and Chin (pers. comm.) found that rapidly-dried excised embryonic axes of jack-fruit could be dehydrated to moisture contents akin to mature orthodox seeds without loss of viability.

A slow rate of drying of recalcitrant seeds favours the progress of germination to a more advanced stage and, as a result, seeds become sensitive to desiccation and lose viability even if the original moisture content was high (Farrant *et al.*, 1989). However, if dried rapidly before proceeding further along the germination pathway they can be dried to a slightly lower moisture content before viability is lost. Dehydration to above-lethal moisture contents causes sub-lethal damage of seeds which can be repaired by the addition of water; such seeds germinate slowly because it takes time for the damaged cells to be repaired. The stage where additional water is required in recalcitrant seeds appears to coincide with the onset of cell division and increasing vacuolation, and it is with the onset of these events that imbibed,

germinating orthodox seeds also become irreversibly sensitive to desiccation (Berjak *et al.*, 1984; see Farrant *et al.*, 1988).

Desiccation tolerance in orthodox seeds is expressed at the protoplasm level and when rehydrated they have the ability to limit and reverse any damage caused by water loss and retain metabolic integrity: however, rapid water loss is more injurious than slow drying (Berjak *et al.*, 1990). According to King and Roberts (1980), orthodox seeds have lower moisture contents than what is required for germination when they fall from their parent plants, while recalcitrant seeds are fully imbibed and capable of immediate germination. The latter are normally shed at water contents in excess of 80% and can withstand the loss of water to approximately this level. Between 80 and 28% water content sub-lethal damage can occur - this is more tolerated with rapid than with slow drying (Pammenter *et al.*, 1991). A high proportion of water in the cell is associated with large molecules, proteins, and sub-cellular structures (vicinal form) and it is this vicinal[1] water which has a role in maintaining the stability of these structures (Berjak *et al.*, 1984). The level of vicinal water is higher in recalcitrant seeds because there is no decline in metabolism associated with maturation drying as in orthodox seeds.

Some investigators have established certain formulae in which seed moisture content or other parameters can be predicted. According to Ellis *et al.* (1991a), orthodox seeds are those which not only tolerate desiccation but whose longevity is thereby improved in a predictable way. Hence, the seed viability equation,

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[1] The concept of water closely associated with macromolecular surfaces having a structure imposed upon it, is now widely accepted and the concept and some of the biological consequences have been reviewed (Drost-Hansen, 1971; Drost-Hansen and Clegg, 1979) cited in (Berjak *et al.*, 1984). The properties of this structured or vicinal water are considered to be rather different from those of bulk water. The cellular microtrabecular lattice (Porter and Tucker, 1981) would increase the surface area available for imposing structure on cell water, and it is probable that a high proportion of water in a cell is of the structured, vicinal form. Such structuring would allow for the ordering of 'soluble' cytoplasmic components such as the multi-enzyme systems of a cytoplasmic metabolic pathway. Furthermore, the presence of vicinal water associated with subcellular surfaces and macromolecules may be important in maintaining the stability of these structures (from Berjak *et al.*, 1984).

S_L = antilog (6.49 - 0.269 m - 0.052 T_E)

where

S_L = Storage Life (days)

m = moisture content (% wet basis)

T_E = Effective Temperature (°C)

is used to predict the life span of the seeds in store. As temperature falls, the recommended moisture content rises; e.g. at 30 °C the moisture content is 9.6% and at 10 °C the recommended moisture content is 13% (O'Dowd and Dobie, 1983).

Similarly, a Seed Survival curve could be formulated as follows:

$v = K_i - p/\sigma$,

where

v = *probit*[2] of percent viability

p = storage period (days): indicator of longevity

K_i = constant, the initial % viability (probit value)

σ = standard deviation of distribution of seed deaths with time.

The genotype and pre-storage environment (conditions during ripening, harvesting, drying and processing) affect the value of K_i but not σ, whereas the storage environment (temperature and moisture contents) affect the value of σ but not K_i (Roberts and Ellis, 1984). Viability may therefore be affected by pre-storage as

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[2] Probit analysis is the transformation of percentage or proportion of experimental results whereby a straight line is obtained. A slope of the line can be calculated to predict on the average, the probit values for the line for any values of the treatment on the x-axis. A normal sigmoid curve is transformed to a straight line when the ordinates are measured on a linear scale of probits instead of percentages (Finney, 1971). The probit value is positive when probability, P > 50%, 0 when P = 50% and negative when P < 50%. In the seed storage experiment, seed death is normally distributed over time and hence a sigmoid curve can be transformed to a straight line on a probit scale. The time it takes for the seed to decline down to 50% viability can be predicted since this percentage is 0 on probit scale.

well as storage conditions. According to Wang (1988), the quality of seeds is affected by four main factors: seed harvesting, cone handling, seed processing and seed storage. Similarly, Adam *et al.* (1989) claimed that seeds collected from the upper portion of the canopy exhibited higher viability than those from the lower portion, perhaps because of less photosynthesis of the latter which had more environmental stress during their relatively long stay on the parent plants.

2.2 Physiology of seed desiccation

Seed maturation and drying are important and in many cases a prerequisite for physiological development. Certain chemicals are involved in seed maturation during embryo development: abscisic acid (ABA) appears to be important in maintaining embryogeny and suppressing germination during the phase of rapid embryo growth preceding desiccation Finkelstein *et al.* (1985) but Tompsett (1984) showed that seeds of four of the *Araucaria* species (*A. araucana, A. angustifolia, A. hunsteinii* and *A. bidwilli*) cannot be safely dried below 25-40% moisture content, just fitting into the storage physiology groups described by Roberts (1973), and these are thus of the recalcitrant type. It should be noted that desiccation has an effect on tissues as some of the water is part of the tissue structure. Desiccation may have a metabolic effect on the cell and metabolic activity - especially enzymes of the dehydrogenase group - has been shown to be closely correlated with seed viability (Hanson, 1984). These enzymes are involved in many metabolic reactions and their failure could cause loss of seed viability or non-germinability of the seed. The structural and physiological factors that make seeds sensitive to desiccation are poorly understood. Hanson (1984) suggested that three areas of damage may be involved (i) cell membranes, (ii) enzymes and hence protein synthesis, (iii) chromosomes. Solute leakage from tissues is generally considered to be a good indicator of structural damage.

Protein structures resist and tolerate high temperatures during desiccation of seeds. The ability of desiccated systems to withstand high temperature is a consequence of the increased resistance of proteins to denaturation upon dehydration (Leopold and Vertucci, 1986). Tolerance to high temperature is due to the

immobilisation of protein structures as the hydrogen bonds are lost; at low temperature denaturation is due to the lack of sufficient heat to avoid freezing damage to the seed which occurs at water contents of above 35%.

According to King and Roberts (1980) the reason for a fall in viability with declining temperature (in, for example, cacao, *Theobroma cacao*) could be:

1. the presence of some temperature dependent reaction, the cessation of which causes lethal disruption;
2. the absence of some protective substances which is present in chilling-tolerant seed;
3. liberation of certain toxic substances to cold-induced changes in membrane permeability.

Ibanez and Casa (1956b) in King and Roberts (1980) also pointed out that seeds of cacao were killed by a factor released at low temperature and this factor could be destroyed if the seed is subjected to heat, showing apparent reversibility. However, it would appear that it is the central region of the seed or embryo axis which is particularly susceptible to damage, and this depends on the period of exposure to low temperature as only the outer layer of the seed will have a chance to cool in short periods.

2.3 Seed injury

2.3.1 Desiccation and temperature

Many investigators have dealt with causes of seed injuries and recommended possible solutions. For example, although the excised embryos of oil palm (which is assumed to be recalcitrant) resist desiccation, this does not protect against lethal injury after prolonged exposure to -18 °C where the injury could be due to intracellular freezing or the extreme cellular dehydration that can occur as a result of extra-cellular ice formation (see Grout *et al.*, 1983). According to Hanson (1984) storage of desiccation-sensitive seeds at sub-zero temperatures is not possible because ice crystals are formed within the cells resulting in damage to organelles and membranes, and the higher the moisture content the greater this problem. On the

other hand, freezing temperatures do not harm desiccation-tolerant seeds because supercooling prevents the formation of ice crystals and also the endosperm may be a barrier to the internal propagation of ice crystals from outside, as in lettuce for example. Bradbeer (1992) cited air-dried seeds showing great resistance to long exposure at temperatures between -20 and -196 °C. Many tropical seeds are killed because of chilling injury if stored between 10 and 12 °C, perhaps these species have never been subjected to selection pressure for this character (Hanson, 1984). Furthermore, some tropical seeds (e.g. cacao and mango) cannot withstand temperatures lower than 10 °C (Roberts and King, 1980), or 10-15 °C (Fu *et al.*, 1990). Newmarch (1979); Tompsett (1981) cited in Ezumah (1986) reported that cold storage has an adverse effect on the viability of neem (*Azadirachta indica*) seeds which do not store well at 6-11 °C.

In some cases, seeds should be pre-chilled before rehydration. For instance, Jones *et al.* (1991) reported that seeds of noble fir need to be moist to respond to pre-chilling and to this end, ways to achieve rehydration which avoid immersion in water have to be investigated. Some researchers use a hydration-dehydration technique to maintain the life span of seeds (Chin, 1988). Similarly, Ponnuswamy *et al.* (1991) recommended a hydration-dehydration treatment (with 10^{-4} M disodium sulphate) during storage for prolonging the viability of *Ailanthus excelsa* for example. Seeds which are hydrated, dried back to their original weight and rehydrated did not suffer impaired germination or vigour, provided the dehydration was performed prior to the transition from a desiccation-tolerant to a desiccation-sensitive state, which coincided with the initiation of radicle elongation (McKersie and Stinson, 1980).

Koster and Leopold (1988) reported a loss of desiccation-tolerance with an increase in reducing-monosaccharide content, and the accumulation of these reducing sugars in a drying seed could lead to the occurrence of the Maillard reaction in which protein and nucleic acid damage threaten the viability of seed. The mechanism of protein and nucleic acid damage should be known. During desiccation, monosaccharides or sucrose in the non-crystalline form react with the seed's cell membranes and the hydroxyl group of sucrose replaces structural water, a reaction which cannot be reversed. Bewley (1979) agrees with the concept that an embryo

starts to develop during imbibition and reaches a desiccation-sensitive phase when irreversible changes occur on subsequent drying.

Several researchers have reported that chilling can cause physiological damage to seeds. The decline of membranal lipid fluidity during chilling results in changes of membrane thickness, membrane permeability, cation concentration and the consequent alteration in the conformation and the activity of enzymes which can be lethal to seeds (King and Roberts, 1980).

2.3.2 Micro-organisms and physical damage

Seeds stored for long periods can undergo physiological changes and may also be attacked by micro-organisms and/or insects. Lesions arising in the aleurone layer of aged seeds, in addition to those found in embryo tissue, may have a significant role to play in loss of viability during storage (Livesley and Bray, 1991). Although physiological changes of seeds accompanying physiological ageing and attack by storage fungi (primarily certain *Aspergillus* species) have been catalogued, the basic biochemical events leading to these changes are not known (Harman and Mattick, 1976)). Storage under high temperature, moisture content and oxygen tension causes aging of the seeds resulting in membrane damage which increases the risk of infection by microflora and also of imbibition injury (Pandey, 1989). Warham (1986) considered it possible that damage to and deterioration of seeds for commercial use is greater in tropical than temperate regions because (1) mechanical, transported seeds are subjected to bouncing and vibration on the poor roads, (2) biological, because of insect infection, micro-organisms attack the seeds through the holes drilled by insects, (3) climatic, that there are prolonged and frequent precipitation, high humidity and temperature.

The problem of fungal association with seeds has to be solved by regular checking of samples in the laboratories. According to Hewett (1987) some seed-borne fungi maintain their identity after many years of cold storage of seeds and hence all accessions should be tested on receipt or before distribution from storage. To maintain viability freshly collected recalcitrant seeds should be exposed to hot water, a fungicide, or both to inhibit the activity of micro-organisms, and be kept in a humid

environment just below full hydration by enclosing them in a thin polyethylene bag which is closed but not sealed to allow gas exchange (oxygen is required in moist storage whether seeds are recalcitrant or orthodox) (Roberts *et al.*, 1984).

Researchers recommend various storage media for seeds, the type being commonly based on the character of seed species. Roberts *et al.* (1984) recommended the media to be moist and inert in a cool condition (e.g. charcoal or sawdust), or in a closed container over an appropriate saturated salt solution (e.g. copper sulphate) or osmotica (such as solutions of polyethylene glycol to control water potential). For orthodox seeds air-tight containers result in a high CO_2/O_2 ratio, regarded as a favourable condition to prolong the life span of seeds (Thapliyal *et al.*, 1991). For example, bamboo showed better germination after this type of storage at 6.6% moisture content. The most successful method of storing recalcitrant seed is by sealing the seeds in polyethylene bags, which reduce water loss and permit gaseous diffusion, provided microbial growth is kept to a minimum (King and Roberts, 1980). Thus *Coffea arabica* has been maintained for 2.5 years at storage conditions of 41% moisture content and 15 °C. However, it is also reported that the optimal storage condition for *Coffea arabica* is close to 10-11% moisture content and 10 °C (Hong and Ellis, 1992a). Metal cans and glass bottles with tight-sealing threaded caps and good quality laminated aluminium foil can be recommended as watertight. Other research has shown that polyethylene was only moisture proof at low temperatures, cotton paper bags absorbed the greatest amount of moisture and where levels exceeded 14% there was a possibility of ice crystals forming within the tissues (Freire and Mumford, 1986). Because of the high moisture content needed for recalcitrant seeds aeration is essential since respiration would otherwise reduce oxygen concentration and eventually shorten the storage life (Roberts and King, 1980; King and Roberts, 1980; Tompsett, 1984).

The problem of recalcitrance remains with us for some time unless a great diligence is shown in researching into the biology and the physiology of those seed species. The abnormality of recalcitrant seeds is not only that they are sensitive to dehydration but also that they shed seed before moisture is reduced and before the intracellular compartmentation, characteristic of seed maturation, is complete. It is

likely that the inability to produce a certain polyunsaturated fatty acid causes this peculiar behaviour (Perl, 1988): there is a concomitant loss of mitochondrial integrity with the loss of water by recalcitrant seeds and as a result a decrease in respiratory enzymes (Nkang and Chandler, 1986).

Furthermore, Berjak *et al.* (1984) in Farrant *et al.* (1988), suggested that recalcitrant seeds lack the mechanism of allowing drying to low moisture contents; this may be partially due to the inability at any stage to form membrane structures capable of stability in a water-depleted micro-environment such that when these seeds are shed they have sufficient water to commence germination. However, many recalcitrant seeds have well developed seed coats which are membranous and impermeable to water, larger in size and heavier (Chin *et al.*, 1984).

Physiological membrane alterations and their effects have been studied by several researchers. Damage to the cell membrane or phospholipid causes leaching of seeds. During storage, autoxidation involves the production of free radicals from unsaturated fatty acids and these radicals may then damage cellular membranes, reacting with macromolecules or form hydroxides (Gorecki and Harman, 1987). However, it may be possible to use alpha-tocopherol as a reducing agent to quench the free radicals. Membrane alteration has been observed when seeds are desiccated below a minimum accepted level, involving a lipid phase change from a lamellar to a hexagonal structure (Becwar *et al.*, 1982).

It has also been noted that chilling, freezing and desiccation injury of seeds may all be connected with changes in membrane permeability. Today, an attempt at using cryoprotectants is coming to a success for some seed species or explants. For example, when the cryoprotectant dimethylsulfoxide (DMSO) was used for potato seeds and embryonic shoot explants at -196 °C (liquid nitrogen) and 33.4% moisture content, 86% of the seeds were still capable of germination (Grout, 1980 in Roberts *et al.*, 1984). Similarly, long-term storage of some species like *Shorea roxburghii* and *Mangifera indica* is possible as long as germination blocking methods are devised without leading to excessive dehydration or risk of chilling injury (Corbineau and Côme, 1988), and two such approaches could be by using solutions of suitable

osmotic pressure and cryoprotective agents to enable seeds or seedlings to withstand lower storage temperatures.

In some cases, different parts of a seed may have different moisture contents (e.g. embryos/gametophyte and seed coat in *A. gracilior*). Since embryo condition is the main determinant of seed viability its moisture content should be considered more important than that of surrounding tissues and it may be necessary to store the excised embryo separately. The use of normal low temperature (0 to -20 °C) may not enable storage of seed embryos for the desired period of time. For example, the use of conventional, low temperature seed storage techniques is inappropriate for excised embryos of oil palm (which were assumed to be recalcitrant, although recently shown to have orthodox characters) since after cooling to and storage at the temperature of liquid nitrogen (-196 °C) the embryos do not lose viability (Grout *et al.*, 1983). Likewise, although further work is needed to clarify the responses, *Coffea arabica* is also not truly recalcitrant, because after six months storage at 9% moisture content and -20 °C, viability is still being maintained at its initial value, suggesting that long-term seed storage of this species may be feasible (Roberts *et al.*, 1984).

The genetic depletion in recalcitrant seeds may explain the relatively early shedding process, the lack of membrane organisation to reach the low moisture stage and the lack of ability to withstand low temperature condition (Perl, 1988). Because of a continuous destruction of the natural resources, it is necessary to maintain seeds by an appropriate storage technique. The method should be able to maintain seeds for at least the normal cycle of the plant (from sowing to first seeding). Seed storage in genebanks could aim at three objectives:

1. to be labour intensive,
2. to preserve old genotypes, and
3. to save endangered species (Bradbeer, 1992).

Seeds (particularly recalcitrant ones) might be stored according to the following alternative methods (Roberts and King, 1980):

1. drying and storing at sub-zero temperature for long periods;
2. cryogenetic storage at high moisture content (both for the entire seed or excised embryo axis);

3. traditional techniques - inhibiting germination in storage by applying chemical or osmotic germination inhibitors at ambient or sub-ambient temperatures and at moisture contents slightly below fully hydrated conditions;
4. using far-red radiation;
5. using antibiotics against micro-organism (if necessary);
6. satisfying the probable need for oxygen (but at the same time preventing peroxidation which might alter membrane conformation).

Some researchers do not accept the pre-soaking treatment beyond a certain period of time as it affects seed viability in storage. In seeds of jute (*Corchorus capsularie* and *C. olitorius*), the inhibitory effect of dehydration and water stress could be reduced by pre-soaking for six hours before dehydration, but it is noteworthy that for longer than six hours, pre-soaking may shift the seeds from a dehydration insensitive to a dehydration sensitive phase as a result of increased vacuolation of germinating seed due to increased hydration (Chowdhury and Choudhuri, 1987). Likewise, soaking of *Podocarpus henkelii* seeds beyond two days has a depressing effect on germination causing darkening and loss of viability (Noel and Staden, 1975).

Hard seed coats could be a problem for germination not only by restricting the passage of water and gases but also by physically stopping the embryos from elongation. King and Roberts (1980); Kariuki and Powell (1988) and Dodd *et al.* (1989) pointed out that the epimatium (seed coat) has chemical inhibitors within the endocarp in addition to acting as a physical barrier to germination.

2.3.3 Germination

2.3.3.1 Physiology of germination

Seeds give rise to a new generation of plants, serving to disperse a new generation in space and time eventually providing a form which is tolerant of adverse environmental conditions. Germination is a continuation from the past to a new generation, beginning with the zygote, and the separation of embryogenesis from germination is therefore clearly artificial when considering the development and function of such tissues as the cotyledons of legumes: in the life of a plant, seed

germination is perhaps the most precarious time, and hence, embryogenesis can be viewed as a preparation for successful germination (Dure, 1975). A seed has to be viable and non-dormant to continue a germination process and an orthodox seed needs to be dried after maturation prior to imbibition and resumption of growth of the embryo (Berjak *et al.*, 1990). Desiccation is an important and, in some cases, essential developmental prerequisite for germination of orthodox (poikilohydrous) seeds whereas recalcitrant (homeohydrous) seeds undergo no maturation drying and apparently can not acquire desiccation tolerance.

Generally, recalcitrant species are indigenous to areas with a continuously wet environment, and their seeds are normally shed into tropical forest where sufficient moisture is available for continued germination throughout the year. Zygotic embryogenesis (embryo development) ceases during maturation in angiosperms, and orthodox seeds enter a period of developmental arrest which prevents them from germinating before environmental conditions favour seedling growth (Finkelstein *et al.*, 1985).

In nature, germination of at least some wetland recalcitrant species is by hypocotyl extension and the ability of phasic root production prior to or immediately after seeding provides a mechanism contributing to the survival of such species, particularly where there is a high probability of short-term dehydration (Farrant *et al.*, 1988). The actively growing embryos depend on their food reserves and digested endosperm tissues until the start of food synthesis. De Carli *et al.* (1987) indicated that both sporophytic (embryo) and gametophytic (endosperm) tissues have good reserves at the start of germination. However, the hypocotyl soon exhausts these reserves; together with the radicle it has a high growth rate and the cotyledons, apart from their storage function, can serve in the uptake and translocation of sucrose and other nutrients from the endosperm to the developing organs. Hence the rate of digestion in the megagametophyte is regulated by active loading of the cotyledons and this, in turn, depends on the nutritional requirements of the growing tissue. In Douglas fir, for example, lipids are the major food reserve in both gametophyte and embryo; in such dry seeds the embryo apparently grows at the expense of the

gametophyte with a loss of 8% of the total weight, probably for energy supply (Ching, 1966).

2.3.3.2 Factors affecting germination

For the start and progression of germination, the important factors required are moisture, temperature and oxygen. Light is necessary for most plant species. If seeds are dormant due either to physical restriction of embryo extension or inhibiting chemicals, they will not germinate even if the factors required for germination are met. Some kind of pre-treatment might be required to break dormancy.

(i) Moisture requirement for germination varies between and within species. In fresh developing seed the surrounding tissue has an inhibitory role to germination; following drying (which may be natural or imposed) the same tissue has a promotive effect upon the switch to germination and hence, seed drying appears to play a direct role in post-germinative enzyme production (Kormode and Bewley, 1988). When recalcitrant seeds were subjected to dehydration, several researchers claim to observe an initial process of fast cell division and vacuolation. Berjak *et al.* (1984) pointed out that in the response of the recalcitrant *Avicennia marina* propagules to dehydration, there appear to be two distinct phases - an early stage of enhanced activity, followed by degradative changes ultimately leading to loss of viability and death of cells and it is suggested that this phase of enhanced activity (pregermination events) is a response to time not to seed water content, that occurs despite the dehydration and not because of it.

Seeds have to reach at least a minimum stage of development to be desiccation-tolerant and for resumption of embryo growth (Koster and Leopold, 1988). If they are dried before reaching the desiccation-tolerant stage of maturity or if they are dried after germination has progressed too far they will not germinate. Those which show recalcitrant behaviour germinate at a reduced rate if they are between the required and lethal water content because they have sub-lethal damage due to dehydration which can be repaired, but they do not germinate if they are below the lethal water content (Farrant *et al.*, 1986).

Some seeds are unable to germinate when they are highly dried and because of a big difference in water potential between the seeds and imbibition water. Although it is possible to dry many seeds down to 4 or 5% moisture content without damage it has now become evident that in most, if not all, of these cases, damage occurs not on drying but on rapid imbibition of water when the seeds are subsequently set to germination, and this can be avoided by free humidification (Roberts and Ellis, 1984).

Each seed species has its own optimum moisture requirement for fast development in the germination pathway. There is no electron transport in the first hydration of germinating seeds, photosynthetic electron transport was observed in the second, and both photosynthetic and mitochondrial electron transport were observed in the third 'water-binding' level (Vertucci and Leopold, 1986). Thus, a shift between dormancy and non-dormancy states in (e.g. apple) seeds was observed at least when moisture contents were in or above the binding level II. In *Avicennia marina*, early changes observed in the root primordia are associated with the early stages of germination and in this respect, fresh seeds of the recalcitrant species *Podocarpus henkelii* stored in an air-tight container at a water content of 184% germinated within a few weeks without the addition of any water. King and Roberts (1979) have also found that germination of some other species with recalcitrant seeds does occur in storage (Berjak *et al.*, 1984).

(ii) Temperatures which otherwise hamper metabolism and other cellular activity can have a significant effect on seed germination. The optimum constant temperature for seed germination is generally between 24 to 28 °C (Aiazzi and Argiiello, 1992) and germination is enhanced by alternating temperature between 20 and 30 °C in combination with light-dark cycles. If seeds are to be tested in an alternating temperature regime a decision has to be taken as to which of the two temperatures to consider first (Ellis *et al.*, 1985). If the regime 20 °C/35 °C (16 h/8 h) is taken, two points should be noted. Firstly, seeds are subjected to the lower temperature (20 °C) first and secondly, seeds are exposed to the longer duration (16 h) of the two phases. Similarly, Noel and Staden (1976) considered a temperature regime of 20 to 35 °C for germination of *Podocarpus henkelii* and found the optimum to be 30 °C.

(iii) Oxygen is essential for the building processes in germination. Some compounds located in the seed coat trap oxygen and consequently hamper the embryo from getting access to this gas. For example, prolonged washing of *Cistus landanifer, C. albidus* and *C. laurifolius* seeds and low incubation temperature increased their germination (Corral *et al.*, 1990); this might be related to the presence of phenolic compounds in their seed coats, the removal of which enabled oxygen to reach the embryos. Embryos of continuously soaked *Podocarpus henkelii* seeds developed abnormally, particularly with respect to the radicle and lower part of the hypocotyl; very few succeeded in emerging and this damage is probably due to anoxia, i.e. lack of oxygen (Noel and Staden, 1976).

2.3.3.3 Seed dormancy

Dormancy is associated with seed germination and is one of the problems which hampers the propagation of some plants. However, seed dormancy has its advantages in nature, contributing to the survival of plants. Seeds may be categorised as non-dormant, moderately dormant or deeply dormant, and seed dormancy has been classified as exogenous (physical, chemical, mechanical) or endogenous (morphological, physiological) or various combinations of exogenous and endogenous dormancies (FAO, 1985; Wang, 1988).

Dormancy inhibits germination and it should be noted that when the seeds are not able to germinate due to dormancy this should not be attributed to loss of viability. According to Thapliyal and Nautiyal (1989) the seed pericarp inhibits germination through disallowing water uptake, gas exchange, penetration of light or escape of inhibitors from the embryo, exerting a mechanical restraint to embryo growth or containing an inhibitor. Some endogenous chemicals are also known to inhibit germination: for example, in peanut, dormancy is associated with endogenous ethylene and may be broken with ethephon (Harty, 1983). In some species a slow germination process is normally underway while the seed is still in storage, such seed tested for germination might show results in a shorter time than expected because of its cumulative germination capacity. Recalcitrant seeds such as *Araucaria angustifolia* and *Landolphia kirkii* show enhanced rates of germination when stored

for short periods and then planted out, compared with newly shed seed, as a consequence of the initiation of sub-cellular germinative events (Farrant *et al.*, 1989).

The presence of inhibiting chemicals in the seed coverings is also one of the causes of dormancy: the leachates from seeds are found to cause other embryos to remain dormant (Bradbeer, 1992). Inhibitory chemicals can be effectively controlled by sufficiently chilling seeds until the cotyledon develops sufficient pressure to rupture the seed covering. In a large scale sowing experiment, seeds pre-chilled both before and after storage sown directly (or after rehydration to a moist condition at 12 °C) gave higher and faster germination than seeds which had not been pre-chilled (Muller and Bonnet-Masimbert, 1989). The moisture content at which each species' seed respond to their own specific dormancy-breaking treatment is different. For example, maple seeds, stratified at 5 °C and ≥ 8% moisture content showed increased germination (compared with unchilled seeds) and dormancy was completely broken when the seeds were stored at the same temperature and 11% moisture content (Vertucci and Leopold, 1986).

According to Ojera and Trione (1990) potassium nitrate and gibberellic acid solutions partially prevent the harmful action of 35 °C temperature in the germination process of some seeds. Fully developed orthodox seeds enter into a low viability stage after maturation and because of the presence of abscisic acid (ABA) do not show precocious germination; however, true recalcitrant seeds do not have germination blocks and some are found to be viviparous (Berjak *et al.*, 1984).

Germination is also affected by solute concentrations. Roberts and King (1980) cited the use of osmotic control since solutions of high osmotic potential inhibit germination by physiologically drying the seed. Similarly, Agamy (1986) pointed out that germination of the desert shrub *Zygophyllum dumosum* seed was found to be inhibited when the soil was initially extremely wet (-0.002 MPa), or dry (-0.10 to 10.00 MPa). At lower values of osmotic potential, germination rate is significantly decreased (Thanos and Skordilis, 1987); for instance, at -1.46 MPa germination of *Pinus halepensis* begins after two or four weeks at temperatures of 20 °C and 15 °C respectively.

Light is essential for germination of some seed species. Mayer and Poljakoff-Mayber (1975) in Ladeira *et al.* (1987) classified seeds on this basis as positively photoblastic (light is necessary for germination), negatively photoblastic (germination occurs only in the dark) or light insensitive (germination occurs either in light or dark). According to Hilhorst *et al.* (1986), red light (R) together with (generally potassium nitrate) stimulates the biosynthesis of gibberellins (GA) to break dormancy: the combined action of R and nitrate can be replaced by addition of the gibberellin 4 and 7 (GA_{4+7}). The red irradiated seeds are antagonized by far red (FR) irradiation and the nitrate (KNO_3) acts as a cofactor in the FR-absorbing form of phytochrome. Likewise, some species' seed are unable to produce growth promoting substances and these growth regulators which would otherwise activate germination (Pereira and Maeda, 1986; Okusanya and Ungar, 1983).

2.3.4 Micropropagation

Micropropagation can be achieved:

1. by initiating growth of axillary shoots,
2. through growth and proliferation of existing apical shoots,
3. by induction of adventitious meristems through either organogenesis or somatic embryogenesis, and
4. through multiplication of callus from organs tissues, cells or protoplasts (Mantell *et al.*, 1985).

Biotechnology offers the means not only for rapid and mass multiplication of the existing stock of germplasm but also for the saving of important, elite or rare trees which are threatened with extinction (Bajaj, 1986). One of the best achievements in this technology is somatic embryogenesis whereby large numbers of 'somatic seeds' are produced so that future afforestation programmes can be enhanced.

In the area of plant biotechnology plantlet development involves the formation of embryoids which are eventually transformed into artificial seeds (Bradbeer, 1992). As they lack embryonic coats, they should be covered with suitable materials to protect them from desiccation and to prolong their viability. Ahuja (1993) reported

that in order to develop an efficient 'delivery system' for plants somatic embryos have been encapsulated to produce artificial or 'synthetic' seeds.

Clonal technology is becoming popular in tree breeding programmes. There are many applications, including genetic conservation of endangered species and screening plants for superior quality. The massive destruction of plants for fuelwood and construction purposes could potentially be reversed through reforestation using micropropagation techniques for rapid plant production.

The ability to regenerate quantities of plantlets from somatic embryos of superior trees (wood of better quality, fast growing, improved dimension increment) would improve existing reforestation programmes and be a major asset to the people (Hakman and Fowke, 1987).

Remaining natural forests could be the base for improvement of genetic quality: we should not neglect to make use of the enormous existing potential of genetic variability in the population of most natural forests. While studies on genetic engineering are progressing, breeding and selection on the one hand and genetic engineering on the other can provide exceptional material for clonal material in the long-run (Ahuja, 1993). This technology will fulfil its potential for accelerated genetic improvement on a sustained basis if it is integrated with conventional tree breeding and selection programmes (Timmis *et al.*, 1987).

Clonal propagation has a significant time gain in tree improvement activities. One advantage of using cell culture is its potential for enormous multiplication rates: more than 100,000 plants can be produced from a few 100 ml culture within one culture generation (Mantell *et al.*, 1985; Thorpe *et al.*, 1991; Gupta *et al.*, 1993; Ahuja, 1993). Furthermore, asexual embryogenesis offers several avenues of exploration in higher plants such as protoplast cultures and gene manipulation and the possible use of bioreactors for producing large quantities of somatic embryos (Thorpe *et al.*, 1991).

Tissue culture may expose variation from mutation via somatic recombination and deletion or induction of transposable elements. Somatic embryogenesis, in particular, has become an option for plant propagation in conifers, offering a number of previously unavailable benefits including higher potential genetic gains and their

capture much sooner than is possible from traditional programmes (Franclet *et al.*, 1987; Parfitt and Arulsekar, 1987; Tautorus *et al.*, 1991 and A. John, pers. comm., Forestry Commission, NRS, 1994).

The ability of *Agrobacterium* to be a natural plant genetic engineer could be a very good prospect for tree improvement through micropropagation techniques (Klee *et al.*, 1987). Plant regeneration from protoplasts enables the exploration of direct gene transfer; somatic hybridisation and polyploidisation not only provide a method for producing new hybrids between sexually incompatible species but also facilitate the interspecific or intergeneric transfer of extra nuclear genetic elements (Tautorus *et al.*, 1991).

Another significance of tissue culture is the possibility of cryopreservation. Micropropagation is a potential system of germplasm storage, either as a source of material for cryopreservation or by maintaining materials for protracted periods under growth conditions (Mantell *et al.*, 1985). Embryo suspensor masses (ESM) which are produced through embryogenesis from elite seeds can be cryogenically stored while the rest are further multiplied, mature to embryos, and planted; after five to fifteen years, field performance data are used to select a smaller number of genotypes which are then propagated from cryostored material to meet reforestation needs (Gupta *et al.*, 1993).

The importance of tissue culture techniques in controlling parasitic organisms which attack desired plant species should be noted. Pathogenic microorganisms can be circumvented in micropropagation because of the eradication of these organisms during sterilisation, and there is a lesser chance of infective agents on small explants compared with normal planting stock (Mantell *et al.*, 1985).

2.3.4.1 Somatic embryogenesis

This is a process by which part of a plant is stimulated so as to produce artificial seeds or seedlings. This method of micropropagation is an alternative method of plant multiplication as it is presumed to solve seed shortages in the future. For large scale and economy in seedling production of forest species it is envisaged that somatic embryogenesis may be the only viable *in vitro* propagation method, and one of the

incentives for the use of this method is the potential ease in which artificial seeds will fit into the current seed-based technology (Wann, 1989). In contrast to organogenesis, where shoots and roots differentiate sequentially on different stages, somatic embryogenesis is a one-step process (Ahuja, 1993). According to Boulay (1987) in Thorpe *et al.* (1991), there are several advantages of somatic embryogenesis:

1. it represents methods of obtaining true rejuvenation from mature trees;
2. costs will be reduced compared to adventitious budding;
3. problems associated with field performance of plantlets will be eradicated; and
4. large numbers of somatic embryos can be stored relatively easily in liquid cultures.

Whether by an empirical or logical approach, it is possible these days to initiate plant tissues at the desired type of development. It has been reported that many higher plants have the ability to undergo somatic embryogenesis and the latter therefore represents the fullest confirmation of totipotency (Wann, 1989). The ability to induce pre-embryonic cells or tissues to simultaneously develop cotyledon and radicle ends is a breakthrough in itself. Thorpe *et al.* (1991) pointed out that axillary and adventitious buds lead to plantlet formation via organogenesis through the production of unicellular shoots, while somatic embryogenesis leads to the formation of a bipolar embryo, often similar to zygotic embryogenesis. The ability to induce young seedlings to develop somatic embryoids is another gain in time and inoculation of cultures is easier to do than small zygotic embryos. Initiation of somatic embryogenesis in young conifer seedlings has been reported and an advantage of using germinated seedlings as explants is that labour-intensive procedure of dissecting embryos from seeds is eliminated (Tautorus *et al.*, 1991).

Initiation, maintenance and maturation of embryos must be done in sequence *in vitro*. The frequency of repetitive embryogenesis varies depending on the embryogenic line and the variability inherent in the different stages of the tissue being transferred (Tulecke and McGranaham, 1985). According to Tautorus *et al.* (1991) the induction frequency for somatic embryogenesis from mature zygotic embryos of

Picea glauca was shown to vary with the provenance, seed longevity and the imbibition time of seeds.

The type of embryogenesis depends on the cells or tissues to be initiated, and perhaps on whether or not they go through callus. Hypothetically there are two patterns of development, where somatic embryos are formed from Pre-Embryonic Determined Cells (PEDC) or Induced Embryonic Determined Cells (IEDC). Embryogenic callus is distinctly different from non-embryogenic callus in colour, texture and morphology (Wann, 1989). According to the hypothesis, cloning of PEDCs represents a method whereby embryogenesis can be of direct conversion to proembryos whereas IEDCs develop callus prior to the production of somatic embryos.

Somatic embryos are believed to originate from single cells, whereas organs regenerate through collective organisation of many cells and therefore, plants derived from somatic embryos tend to be genetically alike, while those reared through organogenesis may result in a genetic mosaic (Tomar and Gupta, 1988; Bonga and Aderkas, 1992). However, several researchers claim that somatic embryos usually develop from embryogenetically competent somatic cells *in vitro*.

Additional treatments may enhance the development of pre-embryogenic cells. According to Park and Son (1988) pin-punctured leaves of *Populus nigra* and *P. maximowiezii* were cultured on a medium containing 0.44 µM BA and 2.26 µM 2,4-D for embryogenesis resulted in embryoids.

2.3.4.2 Morphogenesis

In morphogenesis, shoots or roots can be initiated from adventitious or axillary buds and other plant parts with the appropriate growth regulators. Adventitious buds develop better on embryos cultured on media containing 10^{-7} M to 10^{-5} M BA in *Pinus contorta* in a four week period (Arnold and Erikson, 1981). Explants from 50 to 60 year old "elite" trees of *Dalbergia latifolia*, cultured *in vitro* and pulsed with a certain combination of NAA and BA, proved to be very successful in callus induction and shoot regeneration (Rai and Chandra, 1988). Similarly, among the various growth regulators supplemented in the organogenesis and plantlet formations of

Dalbergia sissoo, only BA added singly or in various combinations with IAA, IBA and NAA has shown callus formation and subsequent growth of shoots (Sharma and Chandra, 1988).

Organ formation is also possible from callus *in vitro*. Although direct production of adventitious shoots from embryos is the most effective *in vitro* method of plant production, an alternate approach which is by far the most desirable for the application of genetic engineering techniques is the regeneration of plants from callus via embryogenesis or organogenesis (Noh *et al.*, 1988).

2.3.4.3 Explants

The unique features of somatic embryogenesis in woody species are the type of explant employed, the extended period of somatic embryo development that may involve dormancy considerations and the distinct phenotype of embryogenic tissues (Wann, 1989).

It should be noted that a juvenile plant is the best choice of explant. Juvenile tissues, such as those of the cotyledon and epicotyl, will respond to *in vitro* culture leading to *de novo* or direct organogenesis (Thorpe *et al.*, 1991).

It is generally true that cuttings from lower branches close to the trunk and orthotropic shoots that develop from the base of the trunk are more juvenile than branches from other parts. Total rejuvenation is gained simultaneously for all characteristics during zygote formation in the sexual process and during somatic nuclear embryo formation (Franclet *et al.*, 1987). Roots are very often considered to be more juvenile compared with most parts of trees (Bonga and Aderkas, 1992).

Competent cells undergo morphogenic growth but sometimes neighbouring tissues may hamper the development of these active cells. Bonga and Aderkas (1992) reported that the capacity of cells or tissues to initiate morphogenesis or embryogenesis may be governed by correlative controls exerted by surrounding tissues, and that these might be removed by "miniaturisation", i.e. excision and culture of the apical dome with one or two foliar primordia left attached.

Identification of explants with the highest morphogenic ability is very important, especially for recalcitrant species; and in this respect researchers use markers to

identify the most juvenile tissues, like size, and shape of the shoot apical dome (morphological marker) or the ratio of endogenous auxin to ABA (chemical marker), both of which can indicate juvenility (Bonga and Aderkas, 1992).

Embryo explants using the megagametophyte can be cultured *in vitro* for initiation of organs. Megagametophyte tissue serves either as a nurse tissue or as a buffer to screen the Pro-Embryo-Suspensor Complex from toxic constituents of the media (Wann, 1989).

Both immature and mature embryos have advantages and disadvantages when used as explants. Immature or young embryos have many determined cells to undergo embryonic development and hence are advantageous. The main uses of mature embryos for somatic embryogenesis and reported by Bonga and Aderkas (1992) are as follows:

1. it is difficult to extract seeds from immature cones;
2. mature embryos remain viable for long periods; and
3. immature embryos rapidly lose ABA by leaching if submerged in an ABA-free medium which results in precocious germination.

In seed embryo explants, callus can start developing from one or more parts of the embryo. In some conifers explants from immature or mature seed embryos gave rise to embryogenic calluses formed during the initial 1-3 months of culture and these calluses arose from suspensor or radicle tissue, or cells beneath the cotyledon, depending on the maturity of the explant embryo (Gladfelter and Phillips, 1987). In the same way, Wann (1989) suspensor tissue provides the most viable early-stage proembryos and it appears that embryogenesis is initiated here in *Pinus* and not from epidermal tissue as in *Picea*.

Researchers have been keen to observe whether the megagametophyte or the zygotic embryo was more active in callus initiation. On this point Schuller *et al.* (1989) observed that in the culture of the megagametophyte containing an immature embryo, callus was originated from the zygotic embryo.

Explant sources have also been an area of focus over the last few years. Some investigators have reported the advantages and the disadvantages of using explants from mature trees and the main disadvantage cited was the difficulty of manipulating

them *in vitro*. However, Bonga and Aderkas (1992) listed the reasons for wishing to clone mature trees as follows:

1. some species are male or female sterile, or their seeds might be difficult to germinate (e.g. *Musa*);
2. some species are severely decimated and are threatened with extinction (e.g. *Leucopogon obtectus*);
3. some trees are poor producers of seeds, although they are fertile (e.g. *Tectona grandis*); and
4. in some dioecious species (e.g. *Carica candamarcensis* and *Phonix dactilifera*) only the female trees have economic value; however, propagation by seeds would result in both the desired females and undesired males. Mature trees are therefore selected for micropropagation as they have been in the field long enough to show their superior value.

As to the peak period of bud flush, there may be variations from place to place under natural conditions. In any case, the optimum period for morphogenesis seems likely to be in spring for both conifers and hardwoods.

2.3.4.4 Culture media

In culture media, energy is required for metabolic activities of the tissues. It is apparent that there is an absolute requirement for an exogenous supply of carbohydrate as a carbon and energy source in cultured plant tissues (Thompson and Thorpe, 1987).

Nitrogen plays a very important role in *in vitro* culture just as for any other growing organism. Both inorganic and organic nitrogen increase biomass production markedly, in addition to stimulating the growth of plant cells and tissues in culture (McCown and Sellmer, 1987; Kirby *et al.*, 1987).

Similarly, macro- and micro-nutrients are required in tissue culture media in the right quantities and formulations and ionic strength. Micro-nutrients like Fe, Ni, Cu, Zn, etc are required by plants at least in small amounts for normal growth and development. Many plants could grow on a wide range of mineral salts, others appear

to be specific, and most species/genotypes reveal considerable growth differences when grown on different formulations of micro-nutrients (McCown and Sellmer, 1987).

2.3.4.5 Growth regulators

Cytokinins are generally used for shoot induction in plant propagation activities. Benzyladenine (BA) at a concentration of up to 25 μM is the cytokinin of choice and usually the only phytohormone required, although mixed cytokinins have proved beneficial in some cases (Thorpe *et al.*, 1991). An appropriate combination of cytokinins and auxins initiate embryogenesis and organogenesis.

Auxins are commonly used for root induction and in plant propagation. However, synthetic auxin 2,4-D has often been used for somatic embryogenesis and has been proved to affect the process (Wann, 1989). In the same way, Bonga and Aderkas (1992) claim that somatic embryogenesis in conifers is generally initiated on the same media as organogenesis, with in most cases 2,4-D used as auxin, and Thorpe *et al.* (1991) also recommended auxin, usually 2,4-D or NAA (up to 50 μM).

Abscisic acid (ABA) is involved in maturation of embryos *in vitro* in both angiosperms and gymnosperms (Gupta *et al.*, 1993). By using ABA in the presence of activated charcoal, a suspension culture can be plated directly from the maintenance media to the development and maturation media, without going through regulator absorption or repeated transfers to ABA-containing media: in this way, 50-100,000 somatic embryos per litre of settled cell suspension can be produced with desiccation-tolerance to less than 10% water content.

High Frequency Somatic Embryogenesis (HFSE) and subsequent plantlet development are reported in cultures of *Coffea arabica* leaf tissue by using possible combinations of cytokinins (kinetin) and auxins (2,4-D or NAA). Somatic embryo formation has been observed in a number of secondary or tertiary cultures grown on an induction medium containing reduced concentrations of kinetin and auxin at a high concentration ratio (Söndahl and Sharp, 1977). Sometimes, initiated calluses are transferred to the same, but hormone free media. In embryogenesis of *Picea glauca* and *Picea mariana*, callus initiation was achieved by supplementing the media with

2,4-D (1×10^{-5} M) and BA (5×10^{-6} M); for further development, calluses were transferred to the same media either lacking growth regulators or containing them at a reduced level (5×10^{-7} M 2,4-D) and (5×10^{-6} M BA), incubated at 25 °C (Hakman and Fowke, 1987). Benzyladenine and kinetin can initiate shoot regeneration from callus in combination with auxin, when used at appropriate concentrations (Minocha, 1987b). There should also be an optimum range of concentrations of growth regulators in culture media. The phytohormones 2,4-D and BA have commonly been used at concentrations of 10 and 5 μM respectively, to both induce and maintain somatic embryos of most species (Tautorus *et al.*, 1991). Likewise, the optimum BA concentration for shoot induction in many hard-wood species is recommended (Bonga and Aderkas, 1992) to be between (10^{-6} M and 5×10^{-6} M). According to Scott *et al.* (1988) embryonic axes of *Shorea roxburghii* (timber species) can be used to induce development of axillary shoots when cultured in the presence of BA (0-10 mg/ℓ). Some researchers induce explants with a basal medium in the presence of growth regulators and glutamine. Thus, cotyledons of walnut were cultured on a conditioning medium containing 1 mg/ℓ BA, 0.01 mg/ℓ IBA, 2 mg/ℓ KIN, and 250 mg/ℓ 1-glutamine added to the basal medium which was sterilized for 20 minutes at 121 °C and embryogenesis was observed (Tulecke and McGranaham, 1985).

2.3.4.6 Culture environment

The quantity of agar used in the medium is very crucial in tissue culture. A low quantity of agar or too much water in the culture environment can create high humidity, and in a high humidity environment there is little transpiration. Calcium can not be remobilised in a very humid culture environment and this condition could cause necrosis, i.e. death of shoot-tip (McCown and Sellmer, 1987). According to Gaspar *et al.* (1987), nitrification and high relative humidity in the culture environment could be controlled by taking the right:

1. size and type of container,
2. type of culture vessel closure,
3. type and the concentration of agar (Difco Bacto Agar 11 g/ℓ),
4. climate parameters of the culture room.

Some people consider that gases like ethylene and carbon dioxide promote organ formation in cultures. Shoot forming cotyledons produce considerable amounts of C_2H_4 and CO_2 and the effects of these gases are possibly synergistic and necessary in order for the cytokinin in the medium (BA) to bring about the switch in morphogenesis from the normal maturation of the cotyledons to shoot bud differentiation. However, if excessive amounts of these gases are allowed to accumulate within the flasks after the first 15 days in culture, the process is partially reversed, i.e. dedifferentiation of the shoot buds and browning of the cotyledons at the cut ends (Kumar *et al.*, 1987).

The pH of the culture media also has an optimum range from 5.5 to 5.8 for example. Minocha (1987a) reported that pH has a direct effect on growth media for plant growth cultures; it precipitates certain cations at higher pH values thus reducing the availability of these ions to cells.

Temperature is a very important factor in culture media as it affects the rate of metabolic processes and investigators dealing with recalcitrant tree species should give more attention to temperature (Chalupa, 1987). Researchers use different temperatures depending on the nature of the species. However, there should be lower and upper limits. Incubation temperature for culture activation seems to range between 20 and 28 °C.

Gases may be generated in culture media because of biochemical interactions. Oxygen is available and essential for metabolic activities. However, other volatile gaseous substances (e.g. carbon dioxide, ethylene, ethane, acetaldehyde and ethanol) may accumulate in the culture vessel and influence growth and morphogenesis (Thorpe *et al.*, 1991).

Light energy is required in tissue culture for morphogenic development. The photosynthesis carried out by most *in vitro* cultures is relatively low and hence they mainly depend on an external sugar supply. However, in the light-induced development of structures the receptor system acts as a switching device which sets the morphogenetic process in motion (George and Sherrington, 1984).

The quantity of light energy might be different at different stages of culture development. Murashige (1974) in George and Sherrington (1984) suggested that an

optimum level of light energy (illumination) was 18 μmol photons m^{-2} s^{-1} (stage II) from a white fluorescent tube, while illumination of 54-181 μmol photons m^{-2} s^{-1} was usually necessary for stage III when the plantlets are transferred to an external environment. The light characteristics which most influence growth and morphogenesis *in vitro* are wavelength, flux, density and the duration of light exposure (George and Sherrington, 1984).

3. Gaps in knowledge

As seen in the previous sections, much is known already about seed viability, germination and propagation of particular species, and from this it is clear that species behave quite differently from each other. The species in the present study have scarcely been investigated in a systematic way even though their potential importance is very high. Particular gaps in our knowledge, for the present species include the following:

Afrocarpus gracilior

(i) No studies are available in which the effect of combinations of moisture content and temperature have been investigated with a view to prolonging the viability of seeds.

(ii) Although there are some germination studies the results are widely inconsistent between seed batches.

(iii) No tetrazolium chloride tests have previously been tried, as a means of rapidly estimating the viability of seed in store.

(iv) Studies on rapid micropropagation techniques such as axillary bud induction and somatic embryogenesis are not available.

Ekebergia capensis and *Pygeum africanum*

(i) The reasons for low germinability of these species are not known.

(ii) The effects of various seed storage temperatures on viability have not been studied.

Before devoting time and money to build an infrastructure for collection, handling and storage of germplasm, it is important to investigate the basic properties of the material as far as can be done in controlled conditions. Such studies should enable us to draw up experimental protocols which ensure the maximum probability of success. Once the right conditions have been defined, the procedures can be scaled up for commercial implementation.

Specific objectives of the work

This thesis is divided into eight chapters, each addressing specific aspects of the general problem of conservation and propagation of forest trees in Ethiopia.

Chapter 1: This sets the scene and defines a number of problems to be tackled in Chapters 2-8.

Chapter 2: The objectives here are two-fold.

1. To investigate the causes of low germinability in *A. gracilior, E. capensis* and *P. africanum*, and to determine specifically whether germination is reduced by dormancy or simply by the lack of proper conditions for storage.
2. To investigate the trend of declining viability in seed storage. This is an important practical investigation as it is required to define the way in which seed storage in commercial conditions may be managed.

Chapter 3: The objective here is to estimate the viability of *Afrocarpus gracilior* seeds in storage with various moisture content and temperature combinations using the tetrazolium method, which is independent of germination inhibitors, and to compare the results with those of the corresponding germination tests.

Chapter 4: This chapter is devoted to preliminary studies on seed storage conditions. The objectives here are to investigate optimum seed storage

conditions, using different combinations of moisture content and temperature, to prolong the viability of *A. gracilior*.

Chapter 5: The problem to be solved here is lack of knowledge of how to maintain seed viability and therefore, an experiment was carried out to determine optimum storage moisture content and temperature combination to prolong the viability of *A. gracilior* seeds. Some inadequacies in the germination methodology which were identified in Chapter 4 are corrected in this chapter. This experiment was done on a different lot to that of Chapter 4, so that variations in germination results are studied between the two seed lots. It was considered necessary to compare results of the three storage experiments in Chapters 3, 4 and 5 and to demonstrate their correlation graphically.

Chapter 6: Some of the problems of deforestation of *A. gracilior* could be partially overcome by developing *in vitro* culture and rapid micropropagation techniques for this species and applying them to plant production. Various shoot and root induction techniques reviewed in the previous section are tested on this species to study the responses they produce.
This chapter also deals with the rooting of *in vitro* propagated shoots in *ex vitro* environment under various treatments like rooting hormone and light levels. The response of *in vitro* propagated plants of this species to the natural environment through acclimatisation is also observed. The ultimate objective of the *in vitro* studies is to investigate whether the plants produced can survive transfer to greenhouse conditions and subsequently field conditions.

Chapter 7: Experiments to investigate the possibility of forming somatic embryos from zygotic embryos via somatic embryogenesis were carried out. It was indicated in the previous section that there is potential for use of somatic seeds in modern seed-based biotechnology. The development of

somatic embryogenesis techniques will pave the way for future genetic improvements in this species through gene transfer techniques such as *Agrobacterium* transformation.

Chapter 8: The objective of this chapter is to summarise the main points of the experiment and re-evaluate the points of introduction with regard to the results. The chapter also suggests future strategies for seed procurement and propagation techniques of forest species.

CHAPTER 2

Germination studies on three Ethiopian native species: *Afrocarpus gracilior, Ekebergia capensis* and *Pygeum africanum*

2.1 General Introduction

There is a wide range of ecological zones in Ethiopia, and a corresponding variety of indigenous forest tree species, two of which are conifers (*Afrocarpus gracilior* and *Juniperus procera*). Remnants of the original dry evergreen forest are found in the northern, central and south-eastern zones and humid forest types in the south-western zone (Mesfin *et al.*, 1987; Bekele, 1993).

No clear data are available regarding forest cover before the introduction of mechanised exploitation but several reports indicate a figure of about 40% in the mid 20th century. Today, the overall forest cover is estimated at between 4% (various Government reports) and 13% (FAO, 1995), both less than the mean for Africa indicated in Chapter 1.

Natural forests have been cut down mainly for industrial timber, expansion of farmland, fuel and construction wood, and it has taken a long time for the impacts of clear felling to be recognised. In some places, it is too late for the ecosystem to recover: it is much easier for natural forest to recover from surviving remnants than from bare land.

Some of the attempts to restore forest cover in the last 30 years appear to have accelerated the destruction of natural forest. For example, in some areas planning has been based on complete removal of the non-industrial indigenous species and their replacement by industrial indigenous and exotic species. Thus accessible forests, mainly over-mature remnants of *J. procera* and *A. gracilior* with the few hardly merchantable secondary species such as *Olea chrysophyla, O. hochstetteri, Pygeum africanum, Celtis kraussiana, Ekebergia rueppeliana, Pouteria ferruginea* etc, have been cleared and replaced with *J. procera, A. gracilior, Cupressus* and *Pinus* species according to Haile (1961). Some of the species suggested for reforestation (e.g. *A. gracilior*) are shade demanding and would regenerate better under forest conditions

using selection or shelterwood rather than clearfelling systems. However, many of the secondary species are economically important (e.g. *Ekebergia* spp., Ogata, 1991) and Breitenbach (1963) reported that *Pygeum africanum* is a useful timber for lorry bodies, bridge decking, heavy constructional work, strong furniture and veneers.

From an ecological point of view the mixed forest should be kept intact: the advantages of maintaining the ecosystem in general and getting many other direct benefits are enormous. Mixed forests are also important for shade demanding species such as *Coffea* and young *Afrocarpus*, which become yellowish due to photo-oxidiation of chlorophyll if exposed to full sunlight (L. Negash, pers. comm., Addis Ababa Univ., 1992).

Reforestation programmes have been launched in the last 20 years in an attempt to at least partially satisfy the demands of timber and fuelwood. These programmes have been undertaken mainly by non-governmental organisations in a fixed time period, planting fast growing exotic species. Some reports clearly underestimate the growth rate of the indigenous species: Pohjonen and Pukkala (1990) wrote: "Productive pioneer tree species suitable for plantation forestry have not been found in the Ethiopian flora ... otherwise feasible pioneer species e.g. *Erythrina abyssinica* and *Cordia africana* grow very slowly". However, the fact is, there are many productive pioneer species like *Millettia ferruginea, Croton macrostachys, Albizia gummifera*, and no one doubts the competence of *Erythrina abyssinica* as a fast growing species. It should also be noted that slow growing species can also have valuable qualities such as high wood density, for example.

One of the main consequences of clear felling natural forest is that soils are subjected to large diurnal trends in surface temperature and moisture, which can directly affect the soil microflora and more importantly the soil seed bank, the potential source of forest regeneration. Seeds can stay in the soil for many years until conditions are right for germination and forest cover is necessary to provide suitable environmental conditions for germination of natural forest species (Sevilla, 1987). Some species depend on the soil to maintain seed moisture content (in equilibrium with soil moisture) rather than their own seed protective structures: Noel and Staden (1976) reported that *Podocarpus henkelii* is doubly disadvantaged by its inability to

imbibe rapidly, even from a saturated substratum, and by its inability to control desiccation. The hard seed coat in *Afrocarpus* (Podocarpus) appears not necessarily to give protection against moisture loss as the seed loses water rapidly to about 10% moisture content even during drying under shade.

A major problem of reforestation with indigenous species is propagation. Many forest species have some kind of seed dormancy such as seed coat dormancy (e.g. *A. gracilior*) or embryo dormancy (e.g. *P. africanum*) due to physical or physiological condition. Aqueous leachate from *A. gracilior* was shown to inhibit the germination of wheat seed and retard the growth of its own shoots *in vitro* and similar effects were observed with *E. capensis* and *P. africanum*. The germination percentage of seed stored using conventional methods can fall rapidly, (e.g. 70 to 20% over six months for *E. capensis*). The reason for this low germination may be seed dormancy, condition (viability) or environment, and the effects of these factors are readily confused. The purpose of the investigation reported in this chapter is to devise methods that enable the germination of as many seeds as possible.

This chapter describes a seed germination study of the three chosen species under laboratory conditions. Seed was stored for 12 months at a range of temperatures and with the moisture content determined on arrival from Ethiopia, and germination tests were carried out every three months.

The physiology and dormancy mechanism of the three study species have not been investigated although it would be interesting to know what proportion of their seed germinates under natural conditions. It might be equally important to know whether or not animals (e.g. birds or primates) are involved in some kind of coat scarification: all the three species produce coloured fruits that they are attractive to animals but it is not known whether the hard shell passes through the guts of animals.

Objectives

1. To explore reasons for low germinability of *A. gracilior*, *E. capensis* and *P. africanum* seeds.
2. To investigate the trend of decline in viability of these seeds.

2.2 Botanical description

2.2.1 *Afrocarpus gracilior* (Pilger) C.N. Page, comb. nov.

Page (1988) described *Afrocarpus* as follows: "the species of *Afrocarpus*, all endemic to the African continent, have always been in a somewhat anomalous position in the *Podocarpaceae*. Modern treatments define *Podocarpus* as having fleshy receptacles to the female fruit, which the species of *Afrocarpus* do not. In lacking this character, they resemble *Retrophyllum* and most species of *Nageia*, but differ from both these genera quite strongly in vegetative characters."

Furthermore Hair (1963), Hair and Beuzenberg (1958) and Quinn (1970) reported in Page (1988) that the cytological distinction of *Afrocarpus* from other allied genera is also substantial. *Afrocarpus* has n = 12 chromosomes, differing from *Retrophyllum* with n = 10, *Nageia* with n = 13 and the African species of *Podocarpus* with n = 11. Its modern distribution, entirely African (Congo, Ethiopia and Uganda and discontinuously southward to south and south-east Africa and the Cape) would suggest *Afrocarpus* is a relic genus of Gondwanan origin (Page, 1988).

According to Breitenbach (1963) *A. gracilior* is an evergreen dioecious tree attaining 45 m in height, usually growing between 1700 and 2500 m. Male cones are solitary or more rarely in groups of 2-3; fruits are solitary, each terminal on a short, racked or leafy auxiliary branchlet, thinly fleshy or shining, subglobose to ovoid, 10-20 mm diameter, bloomed yellow-green to purple-black at maturity, the whole structure lacking an inflated receptacle, the outer layer of the seed coat forming a hard and woody shell at maturity.

2.2.2 *Ekebergia capensis* Sparrm. (Syn. *E. senegalensis* A. Juss), *E. rueppelliana* (Fres.) Rich. (Breitenbach, 1963)

A tree up to 30 m high, wood varying from very light pink to light brown-grey, fine-grained with conspicuously developed growth rings, soft and light (specific gravity: 0.62); leaves odd-pinnate grouped chiefly near the ends of the branches; flowers white, sometimes faintly tinged with pink, fragrant; fruit fleshy, globose, about 2.5 cm in diameter, containing two seeds (Breitenbach, 1963).

2.2.3 *Pygeum africanum* Hook. f. 1864 (Breitenbach, 1963)

Pygeum africanum (*Prunus africana*) bark

A tree up to 35 m high, bole slim, cylindrical, normally very straight and clean; bark dark-brown; wood heavy (specific gravity: 0.82); flowers small, creamy white, fragrant; fruit red, depressed globose up to 1 cm in diameter, single seeded (Breitenbach, 1963).

2.3 Materials and Methods

2.3.1 *Afrocarpus gracilior*

The seed lot used for germination studies was collected in Lagalencha, Adaba, Ethiopia, 7°5"N 30°30"E, in January 1993. It was depulped and dried in the sun to the moisture content of 10%. Six kg of seed were transported to Edinburgh in July 1993 by air and temporarily stored for 4 months at 10 °C. Germination experiments were carried out at Forestry Commission Southern Research Station, Alice Holt, where an alternating incubation temperature was available.

Six hundred seeds were randomly taken and divided into two groups of 300 each. One group was randomly selected as 'naked' and the second one as 'intact': in the former, the seed coat was split in a vice, carefully removed by hand and discarded, the gametophyte being placed in water temporarily until the seed coats had been removed from all 300 seeds.

The germination medium was Garside Industrial sand No. 60 (0.3 to 0.7 mm) from Camas Aggregates, Bedfordshire. Prior to use, it was heated to about 120 °C

for two hours to eliminate micro-organisms. The sand was mixed with water in the ratio of 1 kg sand to 15 ml water (this ratio had been shown as optimal from several preliminary experiments). A tray 36 × 22 × 6 cm was half filled with the medium. One hundred seeds were sown in the sand in each tray at a depth of about 2 cm, both treated and intact seeds being sown in the same way. Each of the three trays of naked seeds were randomly allocated to germination temperatures of 20°, [20/30° (alternating 16/8 h)] and 30 °C, and similarly the three trays of intact seeds. The trays were placed in large plastic bags with the open ends folded and lightly sealed with a clip to maintain humidity, and kept at constant temperature in incubators. Under these conditions, moisture was retained for the duration of the experiment. Assessments were done every week for six weeks and germinated seeds were recorded and removed.

2.3.2 *Ekebergia capensis*

Two kg of seed were collected in Arbagugu, Ethiopia, 8°40"N 40°20"E, in May 1994, transported to Addis Ababa and temporarily stored at Forestry Research Centre. The mesocarp was depulped while it was still fresh and the seed was allowed to dry in the sun to a moisture content of 21%. It was stored at a temperature of 10 °C before it was transported to Edinburgh by air in August 1994 where it was stored at 4 °C in the Darwin Building.

Part of the seed was taken to the Forestry Commission's Alice Holt Research Station, where *nicking* and *temperature* experiments were done. The remaining seed was left for one year in the store at 4 °C so that longevity of the seed was measured by germination tests every three months.

In the factorial experiment of *nicking* and *temperature* three temperatures [20°, 20/30° (alternating 16/8 h) and 30 °C] were selected for incubation. One hundred and twenty seeds were randomly taken from the population and nicked with a pair of scissors on the side opposite to the position of the embryonic axis. Twenty seeds were allocated to each temperature, and each treatment replicated twice. The intact seeds were allocated similarly.

The germination medium was Garside sand as mentioned above, prepared in the same way but using 20 ml of water with each kilogram of sand. Trays (21 × 15 × 6 cm) were half-filled with the sand medium: the tray had 14 holes at the bottom for the removal of excess water and gas exchange. Twenty seeds were sown in each tray (about 2 cm below the surface) and each tray was placed in a plastic bag (as for *Afrocarpus*) in the constant temperature chambers.

Assessments were done for germination every week, and the germinated seeds were recorded and removed. Dead seeds were also removed during the assessment period. Here, a seed was defined as 'dead' when an embryo lost robustness and identity as a result of microbial attack.

2.3.3 *Pygeum africanum*

Two kg of seed were collected in Lepis-Arsi, Ethiopia, 7°20"N 38°45"E, in July 1994. The seed was transported to the Forestry Research Centre, Addis Ababa where the mesocarp was removed by hand while the seed was fresh, and dried in the sun to a moisture content of 20%. It was stored at 10 °C in Addis Ababa before transport to Edinburgh by air in August 1994.

Germination tests following the combined treatment of nicking and temperature were undertaken at the Forestry Commission's Alice Holt Research Station using the same methods as described for *Ekebergia capensis*. The remaining seed was stored at 4 °C in the Darwin Building for longevity tests, as above.

Germination assessments were carried out every week and germinated or decayed seeds were removed. Analyses of variance were used to analyse the data of these experiments. The assumptions implicit in ANOVA are: each observation is the sum of effects associated with factors in the experiment, interaction terms, and an error term; and the error term is normally distributed.

2.4 Results

2.4.1 *Afrocarpus gracilior*

As can be seen in Table 2.1, seed coat and temperature play a significant role in the seed germination of this species. The interaction of the seed coat with the temperature was highly significant and its χ^2 value was higher than the effect of the seed coat or the temperature alone (Table 2.2).

Incubation temperature (°C)	Germination (%)	
	Intact seeds	Naked seeds
20	2	1
20/30	8	9
30	0	81

Table 2.1 Germination percentage of intact and naked seeds of *A. gracilior* at three incubation temperatures (n = 100).

Since there was no replication, it was not possible to produce an ANOVA in the usual way. However, Kempthorne (1952) shows that theoretical variance of the transformed proportions arcsin $\sqrt{\text{germination\%/100}}$ is $\frac{1}{4n}$ and this can be inserted in the ANOVA table as an error term with effectively an infinite number of degrees (since this is a known value and not an estimate). F-values likewise have infinite degrees of freedom in the denominator, and are equivalent to χ^2 values: this technique is similar to 'analysis of deviance' (I. White, pers. comm., Forestry Commission, NRS, 1996). In this particular case, the error mean square, $\frac{1}{4n}$(where n = 100) is 0.0025 (Table 2.2).

Source	DF	SS	MS	χ^2	P<
Treatment	1	0.2002	0.2002	80.08	0.001
Temperature	2	0.1953	0.0976	39.04	0.001
Treat*Temp	2	0.4278	0.2139	85.56	0.001
Error (Theoretical)			0.0025		

Table 2.2 Analysis of variance for *A. gracilior* germination data: χ^2 values calculated from theoretical variance.

Figure 2.1 Cross-section and intact seeds of *A. gracilior* (bar 1 cm).

The effect of seed coat, incubation temperature and their interaction are all very significant, ($P < 0.001$, Table 2.2). The χ^2 value of 80.08 indicates that removal of the thick, hard seed coat (which is a barrier between the environment and the gametophyte with an embryo) is very important in germination of *A. gracilior* (Figures 1.2 and 2.2).

Figure 2.2 Germinating *Afrocarpus* seeds after the removal of seed coat (bar: 1 cm).

2.4.2 *Ekebergia capensis*

Results for germination tests

In general, intact seeds germinated better than the nicked seeds, although there was no apparent difference at the alternating temperature of 20/30 °C (Table 2.3). The results showed that this species is sensitive to incubation temperature. The germination at an alternating temperature of 20/30 °C is much better than that at temperatures of either 20 or 30 °C for the nicked seeds. An incubation temperature of 30 °C appears to have been fatal for the nicked seeds but there was no difference in germination between 20/30 °C and 30 °C for intact seeds. However, intact seeds incubated at 20 °C have a relatively high germination percentage.

Incubation temperature (°C)	Germination %	
	Intact Seeds	Nicked Seeds
20	52 (0.81)	27 (0.55)
20/30	37 (0.66)	40 (0.68)
30	37 (0.66)	2 (0.11)

Table 2.3 Mean germination percentage of *E. capensis* (transformed values bracketed), n = 2. Each mean is based on two replicates. For transformed values, the Standard Error of the Difference, SED = 0.095, calculated from the error mean square. Each percentage was based on two boxes of 20 seeds.

Analysis of variance revealed significant differences in germination % between intact and nicked seeds, incubation temperatures and their interaction.

Source	DF	SS	MS	F	P
Treatment	1	0.202	0.202	23.36	0.003
Temperature	2	0.227	0.113	13.12	0.006
Treat*Temp	2	0.163	0.081	9.42	0.014
Error	6	0.052	0.009		
Total	11	0.643			

Table 2.4 Analysis of variance for *E. capensis* germination data.

Seeds of *E. capensis* are illustrated in Figure 2.3. The seed coat appears to have weak points especially in the area of the embryonic axis. In a relatively aged seed (depending on a storage condition) microorganisms grow on the seed coat itself. This could happen even in storage at a temperature of 4 °C. The microorganisms, probably fungi, appear to infect the embryonic axis and the cotyledon. The cotyledon and embryonic axis were green and soon split the coat, allowing the possibility of photosynthesis during germination.


a


b

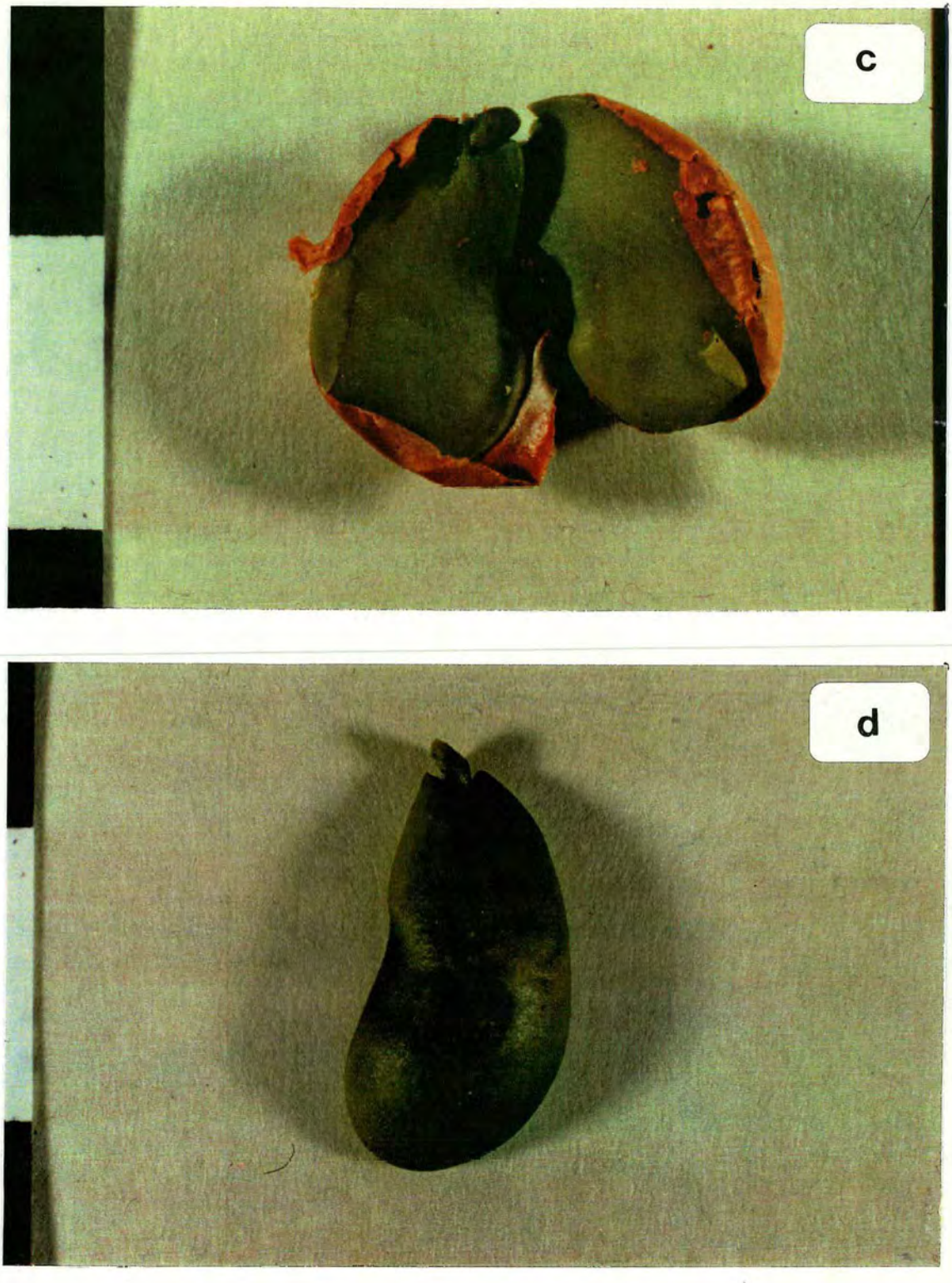


Figure 2.3 Intact, germinating and parts of *Ekebergia* seeds: (a) intact seeds (white bar: 1 cm), (b) germinating seeds (bar: 1 cm), (c) longitudinal section of seed coat, cotyledon and embryonic axis (white bar: 1 cm), (d) naked cotyledon and embryonic axis (white bar: 1 cm).

Results of longevity tests

Viability of the seed stored at 4 °C with a moisture content of 21% decreased steadily over storage periods of up to 12 months to less than 1% (Figure 2.4).

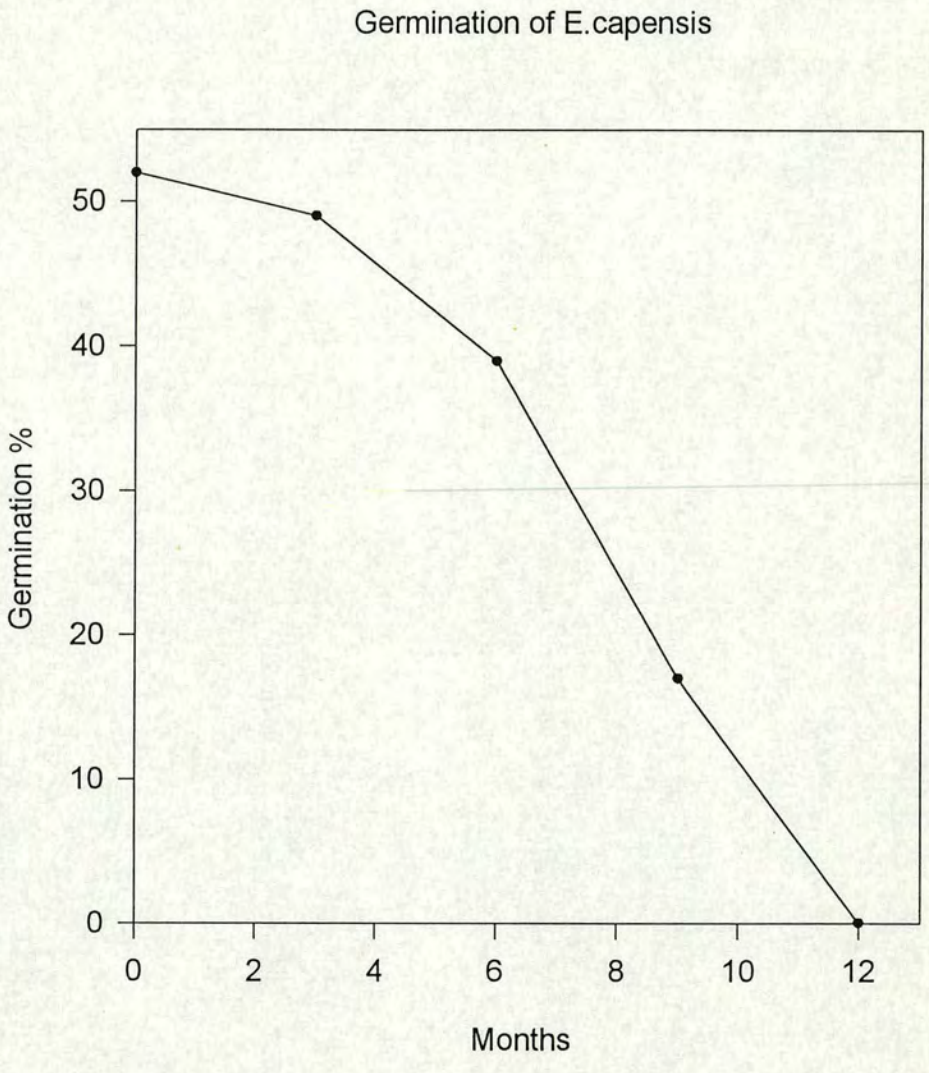


Figure 2.4 Germination of *E. capensis* stored at 21% moisture content and 4 °C over a period of 12 months.

Viability decreased faster after six months in storage. Germination time (germination of 50% of the seed) was initially about three weeks but extended to 4-6 weeks as the seeds aged. Viable seeds imbibe water, extend the embryonic axis and eventually carry the cotyledons out of the germination medium by the extending hypocotyl. Weaker seeds took longer to germinate: they split the seed coat as the radicle protruded form the tip of the cotyledons. Dead seeds were colonised by microorganisms and decayed. The possibility that seed could have tolerated lower moisture contents was not investigated because of time limitation and unavailability of fresh seeds (before being kept in storage conditions).

2.4.3 *Pygeum africanum*

Results of germination tests

It can be seen from Table 2.5 that nicked seeds germinated better than intact seeds, this difference increasing for intact and nicked seeds as the temperature of incubation decreased from 30 to 20 °C.

Incubation temperature (°C)	Germination %	
	Intact	Nicked
20	20 (0.46)	42 (0.71)
20/30	17 (0.43)	25 (0.52)
30	10 (0.32)	15 (0.40)

Table 2.5 Mean germination percentage of *P. africanum* (transformed values bracketed), n = 2. Each mean is based on two replicates. For transformed values, SED = 0.055.

Analysis of variance revealed significant differences in germination % between intact and nicked seeds and between incubation temperatures, although there is no significant difference between the interaction of treatment and temperature (Table 2.6).

Source	DF	SS	MS	F	P
Treatment	1	0.058	0.058	19.14	0.005
Temperature	2	0.102	0.051	16.87	0.003
Treat*Temp	2	0.019	0.009	3.06	0.121
Error	6	0.018	0.003		
Total	11	0.196			

Table 2.6 Analysis of variance for *P. africanum* germination data.

Seeds of *P. africanum* are illustrated in Figure 2.5. The germination rate of *Pygeum* was about 4-5 weeks. The embryonic axis was not visible in unimbibed and freshly imbibing seeds even under the microscope. After about two weeks of imbibition, a developing radicle was sometimes visible in dissected seeds. Protrusion of the radicle occurs close to the hilum and in the middle of the seed, and the epicotyl develops at the base of the radicle. The epicotyl is pinkish at first, soon changing to green (probably at the point when the embryo starts autotrophic life).

Results of longevity tests

Viability was tested every three months. In the first six months of storage there was no significant decrease but viability then fell drastically with no seed germinating after 12 months in storage (Figure 2.6). Some supporting tests indicated that germination after storage at temperatures of -5, 10 and 21 °C was poorer than at 4 °C. Further investigation of moisture content*temperature interaction is required.


a


b

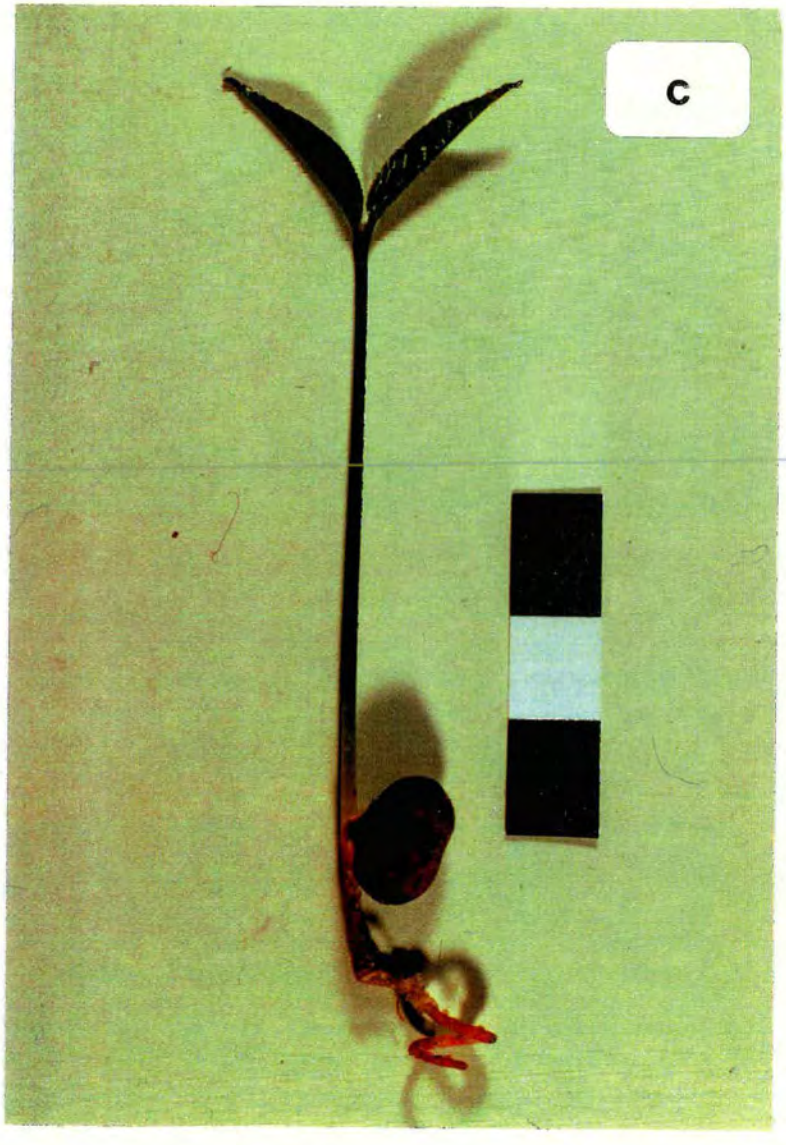


Figure 2.5 Various developmental stages during germination of *P. africanum* seeds: (a) intact and dissected seeds (white bar: 1 cm), (b) radicle and emerging epicotyl at the base of the radicle (white bar: 1 cm), (c) depleted cotyledon, primary root and elongated hypocotyl with a pair of first foliage leaf (scale: 3 cm).

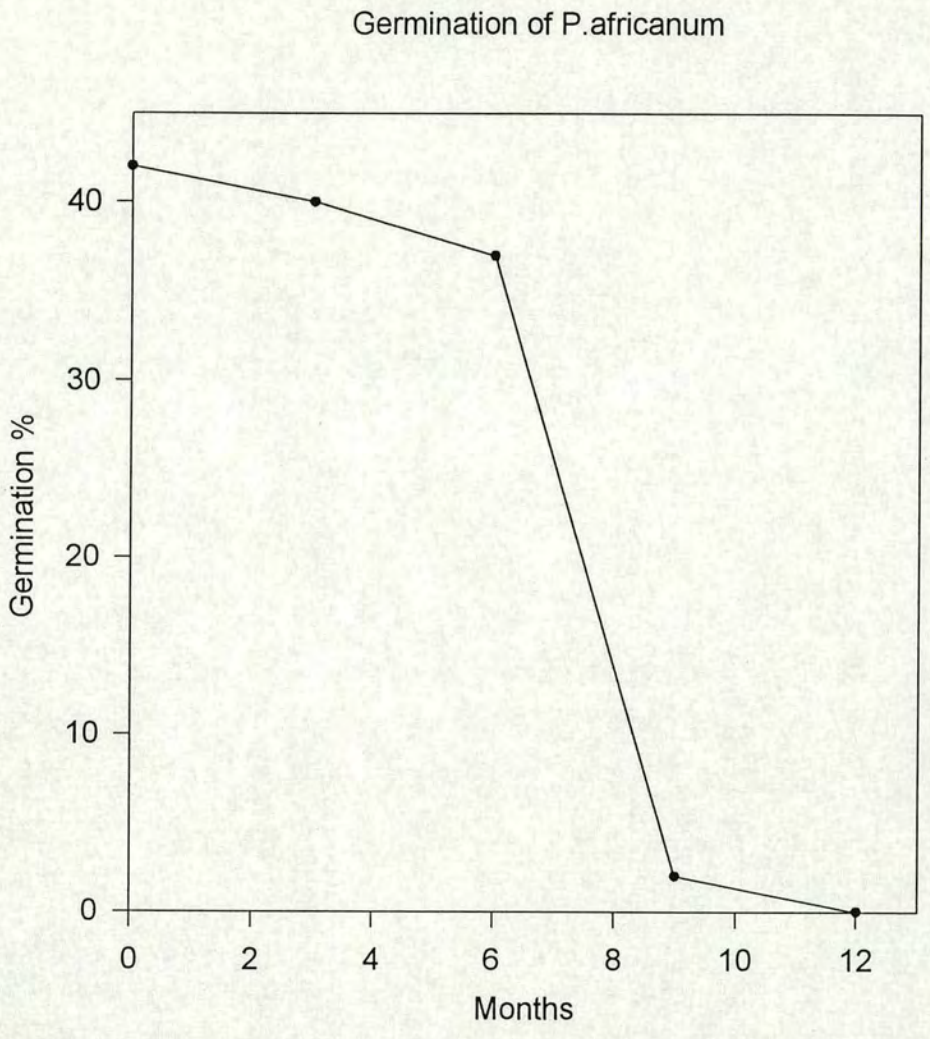


Figure 2.6 Germination of *P. africanum* stored at 20% moisture content and 4 °C over a period of 12 months.

2.5 Discussion

2.5.1 *Afrocarpus gracilior*

Results indicated that the seed coat of *Afrocarpus* is the main cause of poor germination in optimum germination conditions: for example, Noel and Staden (1976) reported that poor germination in *Podocarpus* is imposed by seed coat structures. Intact seeds can take up to six months to germinate and in some cases recorded failure to germinate is due to this low rate of seed development. In initiated and delayed germination, the causes appear to be lack of oxygen (if the medium is too wet) and low moisture. Côme and Tissaoui (1973) cited in Noel and Staden (1976) reported that some embryos remain turgid and alive for several weeks and these embryos develop abnormally, particularly with respect to the radicle and lower parts of the hypocotyl and this damage is probably due to anoxia.

Pre-harvest and post-harvest desiccation might have an effect on germination. Differential changes in the physical and chemical properties of the seed coat during drying could be one reason why different drying strategies have produced varying results with respect to germination capacity (Edwards and Mumford, 1985). Similarly, proper desiccation of developing seeds, whether prematurely or during the final stages of maturation, not only promotes germination on subsequent imbibition, but also results in the cessation of developmentally related synthetic events (e.g. reserve synthesis) and the onset of synthesis associated with germination and postgerminative growth (Bewley and Black, 1994).

Physical maturity of a seed is very important for complete germination. If a seed has not put on mass enough to build cellular organelles (particularly cell membranes) cell contents might leach out resulting in seed death. Anatomical potential, i.e. the percentage of seeds that have the anatomical prerequisites (female gametophyte size and embryo length) for germination when physiological maturity is attained can be calculated to determine the anatomical development (Sahlen and Gjelsvik, 1993).

On the other hand, the requirement of water for germination is simply contact with a film of water. Noel and Staden (1976) reported their studies on *Podocarpus*

henkelii that those seeds from which a slice of the epimatium was removed before planting with the exposed tissue in contact with the moist substratum germinated at 100%, whereas those with the exposed region upwards even with water-saturated air failed to germinate even after six months, indicating the failure to be the result of desiccation.

A. gracilior is sensitive to germination temperature. The optimum germination temperature has been found to be 30 °C in this experiment (see Table 2.1). This agrees with the result obtained by Noel and Staden (1976) on *Podocarpus henkelii*. There was a wide difference between germination percentages for incubation at 20 and 30 °C, as indicated by a significant χ^2 value ($\chi^2 = 39.04$, $P = 0.001$).

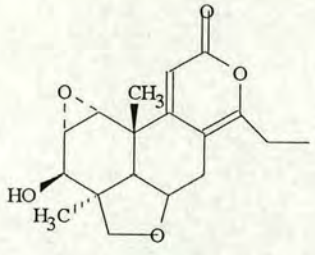
If just enough water, the right temperature and oxygen are available, imbibition results in the expansion of the gametophytic tissues. At the same time, the embryo becomes enlarged and extended. In the presence of the seed coat the pressure which develops as a result of imbibition and vacuolisation can split the coat. The root cap was large and consisted of thick walled cells with large intercellular spaces and since a considerable force must be required to rupture the tough epimatium at germination, it is speculated that the root cap cells may act as a cushion for the emergent radicle (Dodd *et al.*, 1989). This is very similar to what has been illustrated in the photographs, i.e. emerging radicles are tough and thick and presumably are sufficiently turgid to break the seed coat. It can be seen from Figures 2.1 and 2.2 that the proportion of the germinating embryo to the gametophyte is higher after germination has been initiated.

In the initial imbibition, gametophytic tissue enlarges probably until the coat is broken. Although photosynthetic activity begins with the extension of hypocotyl beyond the gametophyte, the embryo still depends on the gametophytic food reserves long after that. As the cotyledons remain in contact with the female gametophyte for up to two months after germination, the mobilization of reserves of the female gametophyte may be considered as part of late germination in *Podocarpus henkelii* (Dodd *et al.*, 1989).

It has been shown in this experiment that germination is either delayed or very poor in the presence of the seed coat in *A. gracilior*. However, whether this delay is

simply due to mechanical restriction of the coat against the developing embryo or to chemical inhibition has not been specifically investigated.

One of the supporting experiments in this work indicated that the organic compound, a diterpenoid lactone called *nagilactone-D*, isolated from an aqueous solution of the seed coat of *A. gracilior*, can completely inhibit germination of wheat seed. This compound has been isolated in collaboration with the Chemistry Department, Edinburgh University, (R.L. Baxter and M. Robin, pers. comm., 1996) as follows: the seeds were ground and extracted with 95% ethanol and then partitioned between organic and polar solvents in an attempt to separate the target from inactive compounds present in larger amounts. This fraction was partitioned by column chromatography method. Repartition and testing the resultants on seed for inhibition continued until this compound was isolated. The minimum concentration for complete inhibition of wheat seed germination has been worked out as 1 mg nagilactone-D per 10 ml water. Its structural formula is as follows:



wheat seed treated with aqueous leachate of *A. gracilior* and the control germinating seeds are shown in Figure 2.7. No experiment was conducted to prove whether or not this compound could inhibit the seed of *A. gracilior* itself during germination because of the unavailability of fresh seed. However, the bulk leachate was applied to growing *in vitro* shoots and shown to retard shoot growth (see Chapter 6 - Micropropagation). It is difficult to say which compound or interaction of compounds was responsible for the result of that experiment.

Kubo *et al.* (1993) isolated antifungal nagilactones (active against *Candida albicans* and two other fungi) from the root bark of *Podocarpus nagi* (Podocarpaceae) and found out that Nagilactone-E, the most abundant dilactone, showed moderate to weak activity against these fungi. As far as this research goes,

the leachate from which Nagilactone-D has been isolated also has inhibited germination of several cereals other than wheat, for example, barley and oats.

Many tree species contain inhibitory substances. According to Khan (1977) there are many substances released when the coat of dispersal units surrounding the embryo (hulls, pericarp, testa, endosperm) is removed, broken or chemically altered, causing germination in both dark and light and suggesting the presence of inhibitors. Del Amo and Anaya (1978) reported that lactones have a specific biological action which depends on concentration, on the type of substance used, and on the species over which it acts.

Figure 2.7 Effect of aqueous leachate from the seed coat of *A. gracilior* on germinating wheat. (a) Wheat seed treated with distilled water, (b) wheat seed treated with aqueous leachate from seed coat of *A. gracilior* (Petri dish: 9 cm diameter).

2.5.2 *Ekebergia capensis*

It appears from the results for this species that the seed coat is not effectively a barrier between the environment and the seed and does not inhibit germination. What appears to have happened is that the seed coat contains microorganisms, probably fungi, which in many cases attack the embryo and the cotyledons especially in moist conditions.

Many seeds in a population have fractures and weak points in their seed coats often in the region of the embryos (see Figure 2.3a). It seems, therefore, that in this species, the seed coat is not completely a barrier to imbibition. It is also unlikely to be a good protector of the internal structure, and micro-organisms appear to invade the embryo through the seed coat itself. Fungi grow even on the seed coats of seeds stored at 4 °C once the seeds have died. This is in agreement with the work reported by Farrant *et al.* (1993) on *Avicennia marina*, who demonstrated that seeds planted with an intact pericarp did not germinate but lost viability, and that there was a concomitant microbial, particularly fungal, proliferation. A supporting experiment indicated that sterilised and decoated embryos appear to have germinated better in *in vitro* conditions than intact seeds where contamination was a problem on an agar medium.

The cotyledon and embryo are green and clear under the seed coat, probably with the potential to photosynthesize soon after seed coat removal in the presence of light (see Figure 2.3 C and D). Intact and viable seeds can germinate in about 2-3 weeks at 20 °C. Lack of viability appears to be the main cause of non-germination. According to Farrant *et al.* (1993), storage within the pericarp severely curtailed longevity and seed death was associated with microbial contamination. This work is in agreement with the conclusion given by Ezumah (1986) regarding neem seed, that members of the *Meliaceae* are short lived.

It is unclear from this experiment which kinds of fungi were associated with contamination during germination. Field fungi and storage fungi are the two general categories which invade seeds. Storage fungi, almost exclusively of the genera *Aspergillus* and *Penicillium*, infect seeds only in storage conditions and are never

present before, even in seeds of plants left standing in the field after harvesting (Bewley and Black, 1994).

It should be noted that the moisture content of 21% in the present work (cf. typical values of 7-8% for oily seeds and 13% for starchy seeds) is sufficiently high to lead us to expect leakage of substances, e.g. sugars, organic acids and amino acids. There is evidence that enhanced leakage causes damping off by fungi (Bewley and Black, 1978; 1994).

Regarding longevity of the seed kept at 4 °C with the moisture content of 21%, viability was almost completely lost in 12 months. Fungal growth was observed on the surface of the coat after a storage period of six months in these conditions. It appears that as long as the moisture content is high enough, fungal growth will occur even at temperatures close to but above zero.

Since stored seeds are more susceptible to fungal attack, it appears that these pathogens are storage fungi. It can be seen from longevity tests (Figure 2.1) that there has been a slow rate of seed death in the first three months, increasing between three and six months and a relatively high rate after six months.

It remains to be seen if this seed stands desiccation to the point below which fungal activity is arrested (normally 8%, as this is an oily seed, 6%) when greater longevity of the seed would be expected.

2.5.3 *Pygeum africanum*

The fact that the seeds germinated better when they were nicked indicates that they have seed coat dormancy. In addition to seed coat dormancy, *Pygeum africanum* appears to have embryo dormancy. The embryos were not visible in the dissected cotyledons and no indication of the embryo was seen under the microscope before about two weeks of imbibition. Germination rate was slow up to 5-6 weeks probably due to the interaction of seed coat and embryo dormancy. The gradual developmental stages shown in Figure 2.5 might indicate that germination is delayed, first by the seed coat and then by slow development of the embryo.

According to Bewley and Black (1994) the embryo itself is sometimes demonstrably dormant (embryo dormancy) in woody species, especially in the

Rosaceae. This is in agreement with the data obtained in this work. Seeds of some species have greatly reduced immature embryos: for example, seeds of orchids have poorly differentiated embryos and other species produce seeds which are much more developed than those of orchids but nevertheless are still immature (e.g. *Fraxinus* spp) where development of the embryo continues after the seed has been liberated from the mother plant (Bewley and Black, 1978).

Seed maturity could also be connected to the level of the inhibitor abscisic acid (ABA). The high concentration of ABA in premature seeds, declining to low concentrations in the mature seeds, coincides with the increase in level and rate of seed germination in mature seeds compared to that in premature seeds (Finch-Savage and Clay, 1994). The physiology of axis development might be explained in connection with the decline of ABA at maturation. Welbaum and Bradford (1990) in Finch-Savage and Clay (1994) explained that the initiation of radicle growth in an imbibed seed may result from solute accumulation and therefore increased turgor pressure, by weakening of tissues surrounding the embryo, or by cell wall relaxation to create a water potential gradient for water uptake. Figure 2.8 displays radicle initiation and development.

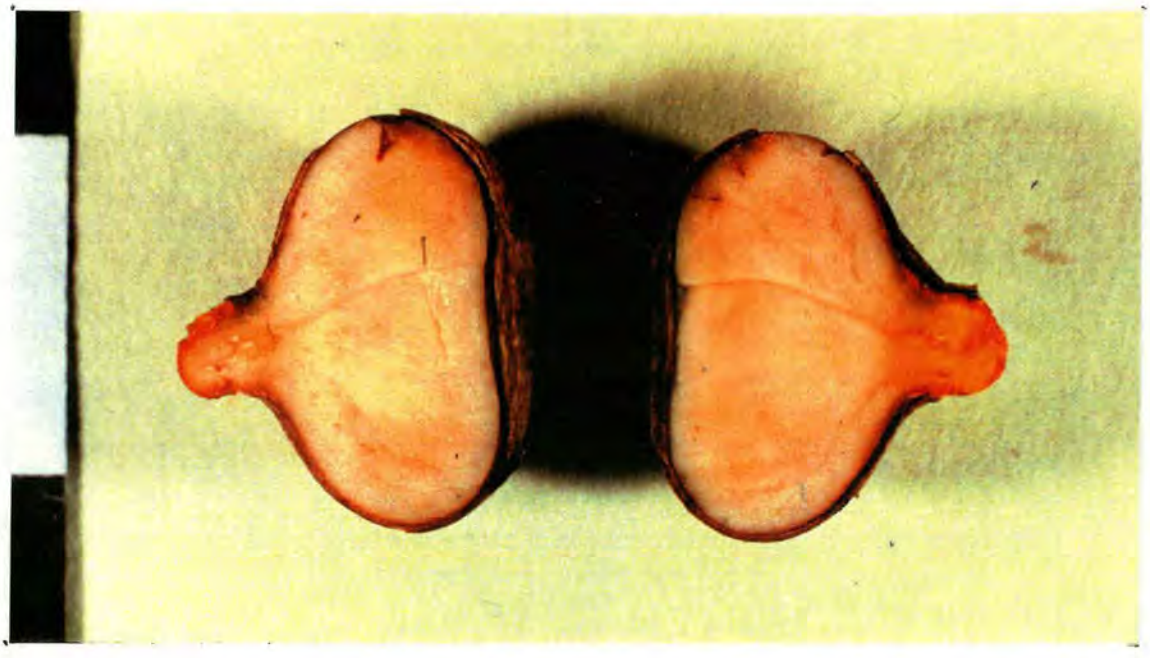


Figure 2.8 Initiation and extension of radicle of *Pygeum africanum* (white bar: 1 cm).

Some reports indicate that the cotyledons themselves might be a constraint to the development of the embryonic axis. Bewley and Black (1994) pointed out that dormant embryos of *Prunus persica* resumed normal growth when the cotyledons were excised at an early stage. Some members of the *Rosaceae* are known to contain inhibitory ABA either in the seed coat or in the embryo itself. According to Bewley and Black (1994) low germinability was associated with the presence of ABA in the embryo of *Prunus domestica* and in the pericarp (testa) of *Rosa canina*.

Prunus africana (*Pygeum africanum*) seems to share many of these characteristics with other members of the family. Longevity of the seed has been measured by a germination method, withdrawing samples from store. Seed stored at 4 °C with a moisture content of 20% has been tested for viability every three months; viability declined only slowly in the first six months but rapidly thereafter (see Figure 2.6).

This result is to some extent similar to that obtained in Kenya on the same species. Shaefer (1990) reported that fresh depulped seeds of *Prunus africana* can be kept in a cold store for at least six months without a considerable loss of germination capacity by mixing them with moist sawdust or peat to maintain high moisture content.

2.6 Conclusions and Recommendations

2.6.1 *Afrocarpus gracilior*

1. The seeds should be mature enough for good germination. Cell membranes of immature seeds are not well developed and this can result in leaching of cellular contents or bursting of the cells.
2. After-ripening and proper desiccation are necessary to level out the differential changes in some properties of the seed. The embryo should be dried to below a certain moisture content (water potential) to reverse the embryonic development to pregerminative processes.
3. Slicing or complete removal of the seed coat is necessary to overcome this major constraint to germination.

4. Incubation medium (e.g. sand) with just enough moisture at 30 °C is the optimum condition for germination. Excess water in the medium can cause anoxia and air circulation in the medium should be ensured.

2.6.2 *Ekebergia capensis*

1. The seed coats of many seeds in the population with cracks in the fusion line around the embryonic axis seem to be a poor protective envelope for the embryo.
2. The proliferation of fungi on the seed coat of seed in store suggests the release of exudates (like sugars and amino acids) from the cotyledons to the surface through the cracks in the seed coat.
3. Low germinability in *E. capensis* is apparently due more to decline in viability than to dormancy.

2.6.3 *Pygeum africanum*

1. *P. africanum* seed has both seed coat and embryo dormancy.
2. It is very difficult to differentiate an embryo in a dissected cotyledon.
3. The fact that immature seeds of some members of the *Rosaceae* contain inhibitory ABA may suggest that *P. africanum* might contain ABA.
4. Nicking of seeds improves their germination rate, and the optimum incubation temperature seems to be 20 °C.

CHAPTER 3

Tetrazolium chloride testing experiment

3.1 Introduction

The 2,3,5-triphenyl tetrazolium chloride ('tetrazolium') test is a biochemical test which differentiates the living and dead tissues of a seed by the presence or absence of a red stain (Sevilla, 1987). Upon penetration into living cells, the salt is reduced to a reddish, water insoluble compound, formazan by electron transfer processes of respiration (Moore, 1985). In some cases viability can be estimated rapidly by employing this test, providing rapid data to indicate the way ahead when planning an investigation of seed germination, for example. Some seeds may not germinate although tetrazolium testing indicates that they are alive but the test is a useful indicator of potential germination capability (Auld, 1986). Tetrazolium results can be correlated with results of other tests depending on the species; in *Acer pseudoplatanus* and *A. platinoides*, tetrazolium staining was correlated with germination results (Dickie *et al.*, 1991) while tetrazolium staining and the 'leakiness' of the cell membranes determined as leachate conductivity were correlated in *Pinus taeda* and *P. caribaea* (Bonner, 1986). There could be two broad reasons for the wide gap between results of tetrazolium and germination tests. One is the lack of experience to interpret what proportion or density of staining will result in germination. Secondly, even when there is a complete tetrazolium staining, conditions for germination may not be optimal, perhaps due to seed dormancy.

The distribution and intensity of the stain should be the criterion used to evaluate the potential for germination and in storage studies the deterioration of the seed can be followed by the difference in staining pattern obtained which is due to physiological changes taking place in a seed as a result of ageing or injury (Raspet, 1965 in Sevilla, 1987). Some researchers have recorded the proportion of staining embryo correlated with germination percentage: for example, Gopal and Thapliyal (1969) compared the results of the tetrazolium test with actual germination

percentages for fresh or stored seed of *Dalbergia sissoo, Bauhiria retusa* and *Acacia modesta*, and concluded that viable seed was reliably characterised by complete staining of the embryo and radicle with > ½ staining of each cotyledon.

According to Sevilla (1987) the accuracy of an individual and his/her efficiency in evaluating a tetrazolium test will depend upon experience, training, dexterity with the fingers, adeptness of the eyes, talent for good judgement of the materials and natural curiosity. However, many other factors can still reduce germination to well below the tetrazolium test figure even if specific treatment is applied to the seed. Roy *et al.* (1984) pointed out in his studies on *Dichrostachys cinerea* that a tetrazolium test indicated 100% viability while the seeds after treatment with sulphuric acid gave germination rates of 63%. Similarly Simak (1970) reported that germination of *Abies alba* tested in sand was 20% while the tetrazolium test figure was 77%. According to Sevilla (1987) tetrazolium staining cannot be impaired by physiological factors and certain chemicals (e.g. 2,4-D) while germination can. According to Leadem (1984) essential growth centres (meristems) must be well stained for a seedling to grow: in the embryonic root the meristem is located near the radicle tip, while in the shoot growth derives from the base of the cotyledons.

The aim of this experiment was to:

(i) estimate seed viability of *A. gracilior* by the tetrazolium method and compare it with viability by the germination method; and

(ii) explore the effect of seed storage conditions on this species.

3.2 Materials and Methods

3.2.1 Seed source and experimental design

The seed of *Afrocarpus gracilior*, lot A2, was collected from Lagalencha, Adaba, Ethiopia in January 1993 and was temporarily stored in one of the local houses in that area. It was transported by an open pick-up vehicle two weeks after collection to the Forestry Research Centre, Addis Ababa. The seed was soaked in water for 3-5 days to soften the outer pulp which was then removed by hand and discarded. The seed was sun-dried and stored at 10 °C in the cold store of the

Research Centre. A sample of this seed was sent to Edinburgh by air at the end of June 1993. It was stored in ambient room conditions at the Darwin Building for two weeks. Moisture content and viability of the seed were assessed at the Forestry Commission's Southern Research Station, Alice Holt as follows: moisture content, 11%; viability, 88% (tetrazolium method), and 81% (germination method) in the middle of July 1993.

As the seedlot was proved to be of good viability, more seed of identical lot was requested from Addis Ababa in order to undertake the storage experiment. This was sent to Edinburgh by courier at the beginning of October 1993 and temporarily stored in ambient room condition at the Darwin Building. Viability of the seed was tested at Alice Holt to be 76% (germination method) and moisture content was determined at Edinburgh to be 11% just when the storage experiment was started at the beginning of November, 1993.

A factorial design of four moisture contents and four temperatures to be tested four times during the storage period of 12 months was decided on after discussions with seed research workers at University of Reading, Royal Botanical Gardens, Ardingly and Forestry Commission Southern Research Station. The moisture contents selected were 6, 8, 11 and 15%, the temperatures taken were -20, 4, 10 and 21 °C and the testing period was every 90 days.

The seed population was thoroughly mixed before any division of the seed was done. The drying process was started by randomly taking 3,840 seeds from the population (this included a surplus of 640 seeds as about 10% of the seed could be damaged and discarded during splitting of the seed coat for tetrazolium chloride tests). The seeds were divided into four moisture content groups of 960 seeds in each. Two groups were randomly selected, one of which was dried to 8% and the other to 6% moisture content using an oven drier at 25 °C with trays containing a single layer of seeds. Each plastic tray (holding 60 seeds) was randomly placed on a larger oven tray so that drying conditions were the same for both seed groups - allocation to shelf level was randomly selected. The seed moisture content was regularly checked by weighing: the moisture content of 8% was obtained after 8 hours but it took 28 hours to reach 6%.

The third group of seed was randomly selected from the remaining two groups and rehydrated to 15% moisture content. This was done in a growth room of 2 × 2 × 3 metres at 90-95% r.h. and at 18 °C. Each of the 16 plastic trays holding 60 seeds was placed randomly on the metallic table in the growth room. The moisture content was obtained by adding the percentage gain in moisture content to the initial moisture content: it took 120 hours to reach 15%. The fourth group of seed was taken as the control and no change in moisture content was made.

Each unit of 60 seeds was sealed separately in a 16 × 20 cm aluminium foil bag with a heat sealer. Each moisture content group had 16 bags of seeds which were selected randomly and labelled with their normal temperature and withdrawal time. Storage was arranged in 4 incubators, each of which received 16 bags (four from each moisture content). The factorial combination of 4 moisture contents, 4 storage temperatures and 4 storage periods gave a total of 64 treatments in this experiment.

Viability tests (tetrazolium chloride method) were carried out at 90 day intervals. It is necessary to make some points on the question of withdrawal. As mentioned earlier, the seed was in storage at 10 °C in Addis Ababa and two viability tests had already been done, of which one was at the beginning of this experiment. Hence, viability of the control seed at the start of the experiment was taken as zero withdrawal although for all treatments no tests were done for the dehydrated and rehydrated seeds at that time. One of the 16 moisture*temperature combinations (11% and 10 °C) was simply the extension of the ongoing storage condition in Addis Ababa. The withdrawal numbers 0, 1, 2, 3 and 4 were allocated to storage periods of 0, 90, 180, 270 and 365 days respectively.

The stored seeds had been organised for tetrazolium chloride test in each withdrawal. Sixteen bags, one for each moisture content*temperature combination, were withdrawn. The seed coats were split with a vice, carefully removed by hand and discarded. The gametophytes were temporarily put in distilled water to avoid desiccation until splitting was finished which took 10-15 minutes to do for 60 seeds.

3.2.2 Preparation of tetrazolium chloride solution

A buffer solution was used in preparing the tetrazolium solution in order to maintain seed respiration which is necessary for the staining to occur. The buffer solution was prepared by taking and mixing compounds according to the following procedure (ISTA, 1985).

1. Potassium dihydrogen orthophosphate (KH_2PO_4), 7.26 g in 800 ml distilled water (acidic).
2. Disodium hydrogen orthophosphate (Na_2HPO_4), 14.25 g in 1200 ml distilled water (basic).
3. The two solutions above were mixed, and this buffer solution was heated to 55 °C.
4. Tetrazolium salt, 20 g was added to the buffer solution. This gave a 1% tetrazolium solution.
5. A 0.5% tetrazolium solution was made by taking half a litre of the 1% solution and half a litre of distilled water.

The seed was withdrawn from the store and the seed coat split (see Chapter 2) after which 50 seeds were taken at random.

3.2.3 Seed dissection, treatment and assessment

All the split seeds were soaked in distilled water overnight to imbibe and subsequently promote respiration. Approximately equal amounts of 0.5% tetrazolium solution were poured into the cells of an ice-maker tray, in two replications of 25 cells. Each seed (gametophyte) was cut half-way longitudinally with a scalpel on a dissection tray (in some cases the two parts were still attached at a single point) and placed in the tetrazolium solution in a cell of the tray. The trays were covered with cling-film and kept at 26 °C in an incubator for 24 hours.

Each part of the gametophyte plus embryo were carefully assessed for the level of staining, being recorded as 'best stained' - completely dark red, 'less stained' - patchy or light red or 'not stained' - beige or unstained.

3.2.4 Statistical analysis

Minitab Release 10.1 was used for data analysis and graphics and Sigma Plot for graphics. Analysis of variance was employed to assess the variation between treatments. Percentage data were transformed into probit values for the following reasons:

(a) ease of calculation of longevity, i.e. the period in storage for seed to lose viability down to a stated level. For example, viability is given by the following equation:

$$v = K_i - P/\sigma$$

where

v = probit of percent viability

P = storage period (days)

K_i = constant, the initial % viability (probit scale)

σ = standard deviation of distribution of seed deaths with time.

(b) a straight line is obtained when trends over time in percentage germination data are transformed to probit values (Finney, 1962; Finney, 1971). It is then possible to get the trend of viability of the seed during the storage period by calculating the slope of this line;

(c) the zero value is equidistant from, for example, 1 and -1 on the probit scale, so that it is easy to remember the reference of 50% viability.

(d) as with some other transformations, probit transformation stretches out both tails of a distribution of percentages or proportions and compresses the middle, making germination data approximate to a normal distribution and this qualifying for use in statistical tests such as analysis of variance.

Raw data were converted to probit values from which analysis of variance and mean viability were calculated. Least significance differences (LSD) were obtained from the error mean square of the analysis of variance. Mean viability was ranked by calculating the difference between any two means and comparing it with the LSD

(Appendix 3.1). In tabulating results, superscript letters (a,b,c, etc) were used to distinguish means that are significantly different (P = 0.05).

3.3 Results

In most cases, tetrazolium values are higher than germination results. It was not possible at this point to demarcate a clear line between viable and dead seeds from the staining. The problem is not only the interpretation of tetrazolium test results but also the difficulty of creating consistently optimum conditions for germination in the laboratory. However, with long experience, the gap between the tetrazolium and germination data could be minimised. In this experiment, the mean percentage viability summarised in the tables refers to 'viable seed' which has been defined as seed which was 'best stained'.

3.3.1 Mean viability

To assess the influence of duration of storage, seed was withdrawn from storage every 90 days for tetrazolium testing.

Percentages of mean viability are summarised in Table 3.1 and analyses of variance in Table 3.2.

In the first withdrawal, significant differences occurred only between the temperature of -20 °C and the other temperatures: moisture content had no significant effect on viability. In subsequent withdrawals both moisture content and temperature, and their interaction, are shown to have a significant effect on viability.

After a storage period of 12 months, seed stored at a combination of 6 or 8% moisture content with temperatures of 4 or 10 °C were found to be most viable.

(a) WD1

Temperature °C	Moisture content %				
	6	8	11	15	$\bar{X}$
-20	58^{fg}	58^{fg}	54^{gh}	34^{i}	51^{d}
4	68^{abcde}	72^{abcd}	68^{abcde}	66^{abcdef}	69^{abc}
10	72^{ab}	74^{a}	74^{a}	68^{abcde}	72^{a}
21	68^{abcde}	72^{abc}	74^{a}	72^{abcd}	72^{ab}
$\bar{X}$	67^{abc}	69^{a}	68^{ab}	60^{d}	

(b) WD2

Temperature °C	Moisture content %				
	6	8	11	15	$\bar{X}$
-20	50^{fghi}	52^{fgh}	50^{fghi}	26^{kl}	45^{cd}
4	68^{ab}	70^{a}	66^{abcd}	66^{abc}	68^{a}
10	66^{abcd}	68^{ab}	64^{abcde}	50^{fghi}	62^{b}
21	54^{fg}	56^{ef}	46^{ghij}	30^{k}	47^{c}
$\bar{X}$	60^{ab}	62^{a}	57^{bc}	43^{d}	

(c) WD3

Temperature °C	Moisture content %				
	6	8	11	15	$\bar{X}$
-20	52^{efghi}	$48^{efghijk}$	38^{jklm}	6^{o}	36^{c}
4	66^{abcd}	68^{abc}	$64^{abcdefg}$	60^{abcde}	65^{a}
10	70^{a}	68^{ab}	60^{abcdef}	44^{ijkl}	61^{ab}
21	$58^{bcdefgh}$	50^{efghij}	38^{klmn}	0^{op}	36^{cd}
$\bar{X}$	62^{a}	59^{ab}	50^{c}	28^{d}	

(d) WD4

Temperature °C	Moisture content %				
	6	8	11	15	$\bar{X}$
-20	46^{efgh}	42^{ghi}	34^{ij}	6^{m}	32^{cd}
4	66^{abc}	68^{ab}	60^{bcd}	52^{def}	62^{a}
10	66^{abc}	70^{a}	60^{bcd}	28^{jkl}	56^{b}
21	54^{de}	48^{efg}	32^{jk}	0^{mn}	34^{c}
$\bar{X}$	58^{a}	57^{ab}	47^{c}	22^{d}	

Table 3.1 Mean viability percentage of *A. gracilior* seed from tetrazolium tests: The significance of a difference between any two means (within any single withdrawal, as each withdrawal was analysed separately) was tested by the least significant different test; means with different superscripts (a,b,c, ...) are those that have been declared significantly different from each other (using probit transformation) at 0.05 level of significance. The column and row *means* were considered separately.

Withdrawn after 90 days

Source	DF	SS	MS	F	P
MC	3	0.27069	0.09023	5.33	0.010
Temp	3	1.70633	0.56878	33.57	< 0.001
MC*Temp	9	0.35393	0.03933	2.32	0.068
Error	16	0.27107	0.01694		
Total	31	2.60202			

Withdrawn after 180 days

Source	DF	SS	MS	F	P
MC	3	1.15611	0.38537	34.54	< 0.001
Temp	3	2.13105	0.71035	63.67	< 0.001
MC*Temp	9	0.35799	0.03978	3.57	0.013
Error	16	0.17850	0.01116		
Total	31	3.82366			

Withdrawn after 270 days

Source	DF	SS	MS	F	P
MC	3	6.10920	2.03640	80.09	< 0.001
Temp	3	4.88441	1.62814	64.03	< 0.001
MC*Temp	9	2.60882	0.28987	11.40	< 0.001
Error	16	0.40684	0.02543		
Total	31	14.00927			

Withdrawn after 365 days

Source	DF	SS	MS	F	P
MC	3	7.26793	2.42264	178.98	< 0.001
Temp	3	4.98185	1.66062	122.68	< 0.001
MC*Temp	9	1.50723	0.16747	12.37	< 0.001
Error	16	0.21657	0.01354		
Total	31	13.97359			

Table 3.2 Analyses of variance for tetrazolium tested viability of *A. gracilior* seed withdrawn after four storage periods.

3.3.2 Withdrawal 1 (90 days)

The effect of moisture content on seed viability in the first 90 days was relatively small but still significant ($F = 5.33$, $P = 0.01$). For moisture contents of 6, 8, 11 and 15% mean viability was 67, 69, 68 and 60% respectively (see Table 3.1a).

The temperature effect was relatively high, with -20 °C giving poor results ($F = 33.57$, $P < 0.001$). Mean viability percentages for -20, 4, 10 and 21 °C were 51, 69, 72 and 72 respectively.

The interaction of moisture content and temperature was relatively low ($F = 2.32$, $P > 0.06$).

3.3.3 Withdrawal 2 (180 days)

The variation in viability between moisture contents was greater than in the first withdrawal with viability at 15% moisture content falling rapidly. The mean viability percentages for 6, 8, 11 and 15% moisture content were 60, 62, 57 and 43 respectively (Table 3.1b). The effect of moisture content was highly significant ($F = 34.54$, $P < 0.001$).

Variation in viability due to the effect of temperature was nearly twice that in the first withdrawal ($F = 63.67$, $P < 0.001$). Mean viability percentages for -20, 4, 10 and 21 °C were 45, 68, 62 and 47 respectively. There was a significant difference between mean viability at temperatures of 4 and 10 °C and further down in the rank order mean viability at -20 and 21 °C was significantly lower than at both 4 and 10 °C.

The interaction of moisture content and temperature was again relatively low ($F = 3.57$, $P = 0.013$).

3.3.4 Withdrawal 3 (270 days)

By the time of this withdrawal, 15% moisture content had increased its detrimental effect on viability especially in combination with temperatures of -20 and 21 °C. Viability percentages for moisture contents of 6, 8, 11 and 15% were 62, 59, 50 and 28 respectively (Table 3.1c). It can be seen from the table that viability decreases with the increase in moisture content. This withdrawal is the point in time when variation due to moisture content has become greater than that due to

temperature, the F-values being 80.09 and 64.03 respectively (both significant at < 0.1% probability).

With respect to temperature, viability was again highest at 4 and 10 °C, with no significant difference between them.

The interaction of moisture content and temperature increased in significance ($F = 11.40$, $P < 0.001$). The trend of viability with regard to moisture content-temperature combinations was by and large the same as in the second withdrawal: at 15% moisture content and a temperature of 21 °C viability was less than 1%. At a temperature of 4 °C combined with any of the moisture contents there was no significant gradient in viability. However, at a temperature of 10 °C there were significant differences between moisture contents (the lower the moisture content the higher the viability and vice versa). Viability was recorded to be highest for the combination of 6% moisture content and a temperature of 10 °C.

3.3.5 Withdrawal 4 (365 days)

The effect of moisture content was again highly significant ($F = 178.98$, $P < 0.001$). Mean viability percentages for moisture contents of 6, 8, 11 and 15% were 58, 57, 47 and 22% respectively (Table 3.1d), the difference between moisture contents of 6 and 8% being not significant.

The effect of temperature remained highly significant ($F = 122.68$, $P < 0.001$): the mean viability recorded for temperatures of -20, 4, 10 and 21 °C was 32, 62, 56 and 34% respectively.

The interaction of moisture content and temperature remained highly significant ($F = 12.37$, $P < 0.001$). There were no significant differences in viability between the moisture contents of 6, 8 and 11% combined with temperatures of 4 and 10 °C. It was noted that at every temperature treatment viability decreased with an increase in moisture content. As in the third withdrawal, viability has been recorded to be very poor at -20 and 21 °C.

3.3.6 Rate of decline in seed viability

Using a Minitab computing programme, slopes 'b' for the decline in seed viability were calculated using the standard regression equation. From the analysis of variance, values of LSD between slopes were calculated by using the error mean square. The slopes were then ranked based on the LSD and tabulated in the same way as the viability percentages (Table 3.3). The mean slope was based on viability values from four withdrawals and from the 'zero withdrawal time' of the control experiment, i.e. seed maintained at Ethiopian conditions of 11% moisture content and 10 °C.

Looking at the results, the moisture contents 6 and 8% maintained seed viability better than the other moisture contents. In the case of the temperature, 4 °C was observed to be the best followed by 10 °C. Results of the interaction between the moisture content and the temperature revealed that 6 and 8% moisture content combined with either 4 or 10 °C produced the best results. The highest slope, i.e. the poorest viability was recorded with a moisture content of 15% and temperatures of either -20 °C or 21 °C.

	Moisture content %				
Temperature °C	6	8	11	15	$\bar{X}$
-20	-1.92^{fgh}	-2.25^{hi}	-2.88^{k}	-6.27^{n}	-3.33^{c}
4	-0.69^{abc}	-0.62^{ab}	-1.26^{e}	-1.60^{efg}	-1.04^{a}
10	-0.70^{abcd}	-0.57^{a}	-1.40^{ef}	-3.51^{l}	-1.54^{b}
21	-1.60^{efg}	-2.28^{hij}	-3.66^{lm}	-7.93^{o}	-3.87^{d}
$\bar{X}$	-1.23^{a}	-1.43^{ab}	-2.30^{c}	-4.83^{d}	

Table 3.3 Mean slope of declining seed viability over time (determined from tetrazolium tests). Slope values with the same superscript are not significantly different at P = 0.05. The column and row means were considered separately.

3.3.7 Trend of viability over time

3.3.7.1 Effect of moisture content

Viability at zero time was that recorded for the control seed at the start of the experiment, a common initial point for all treatments. Thereafter, (Figure 3.1) there are noticeable differences between the moisture contents. For each moisture content the trend of viability appears to divide into two based on the storage temperature, declining less rapidly at temperatures of 4 or 10 °C and more rapidly at temperatures of -20 and 21 °C. Viability trends at 6% moisture content were similar, those at 15% moisture content were much dispersed and hence widely variable and those at 8 and 11% moisture content were intermediate. Generally, moisture contents of 6, 8 and 11% produced viability trends of similar type in the same manner, while moisture content 15% was seen to be detrimental to the viability of the seed.

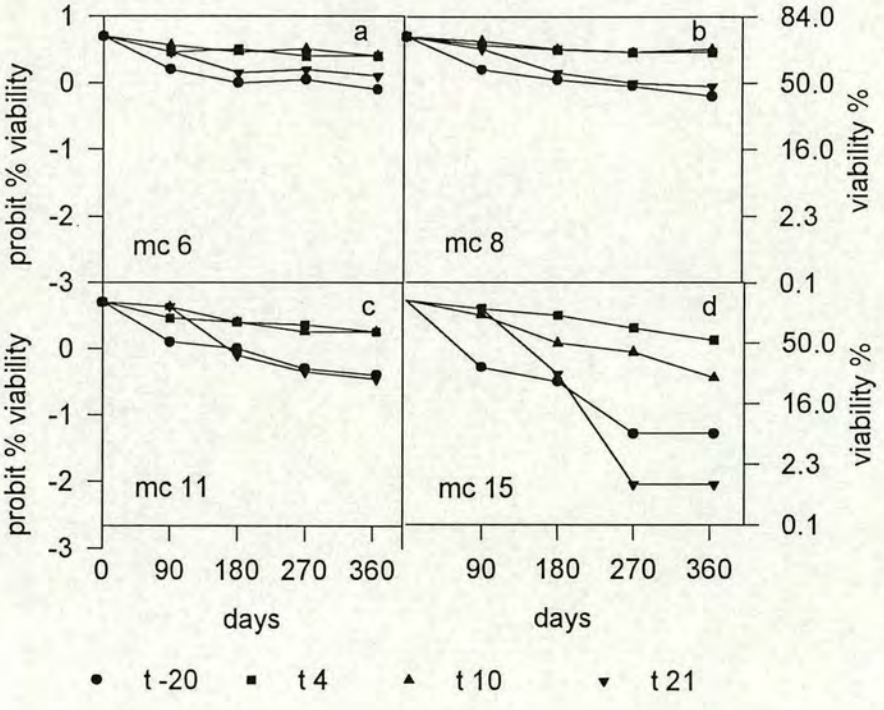


Figure 3.1 Viability of *A. gracilior* seed stored at different moisture contents (a) 6%, (b) 8%, (c) 11% and (d) 15% [tetrazolium test data transformed to probit values].

3.3.7.2 *Effect of temperature*

One effect common to all temperatures, particularly in later withdrawals, was that combinations with 15% moisture content showed poor viability. At temperatures of -20 and 4 °C, there was a noticeable decrease in viability in the first withdrawal compared to the zero withdrawal (see Figure 3.2). Viability declined steadily with time at 10 °C, other than when it was combined with 15% moisture content.

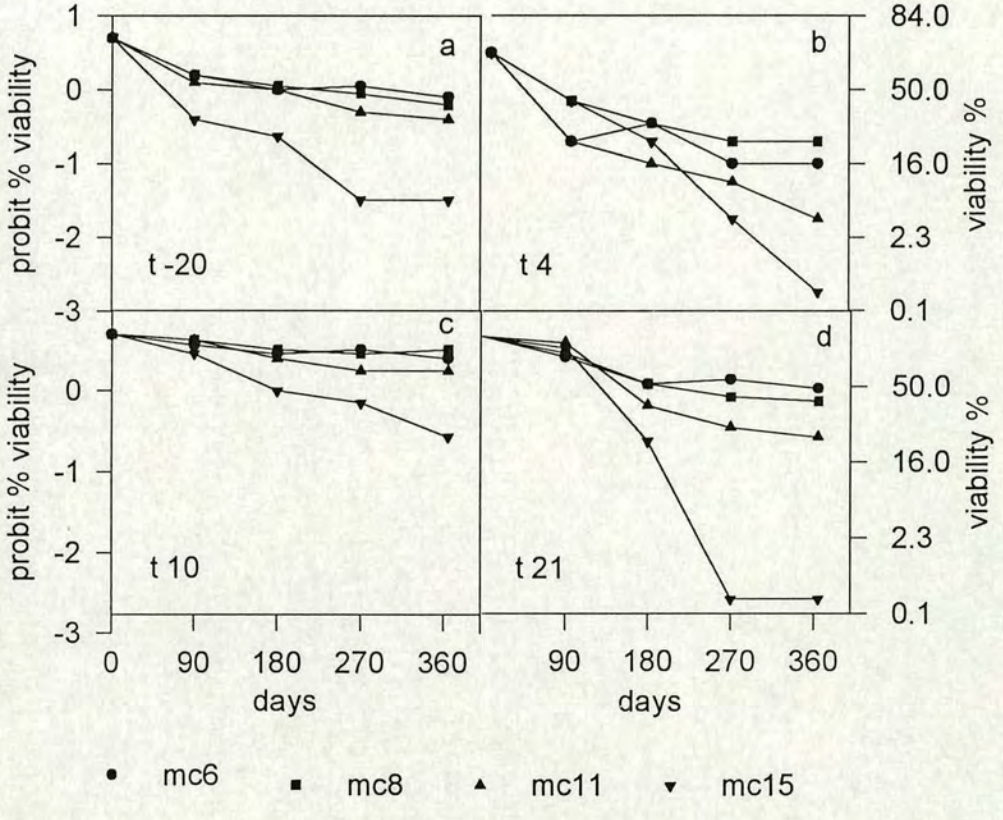


Figure 3.2 Viability of *A. gracilior* seed stored at different temperatures (a) -20, (b) 4, (c) 10 and (d) 21 °C [tetrazolium test data transformed to probit values].

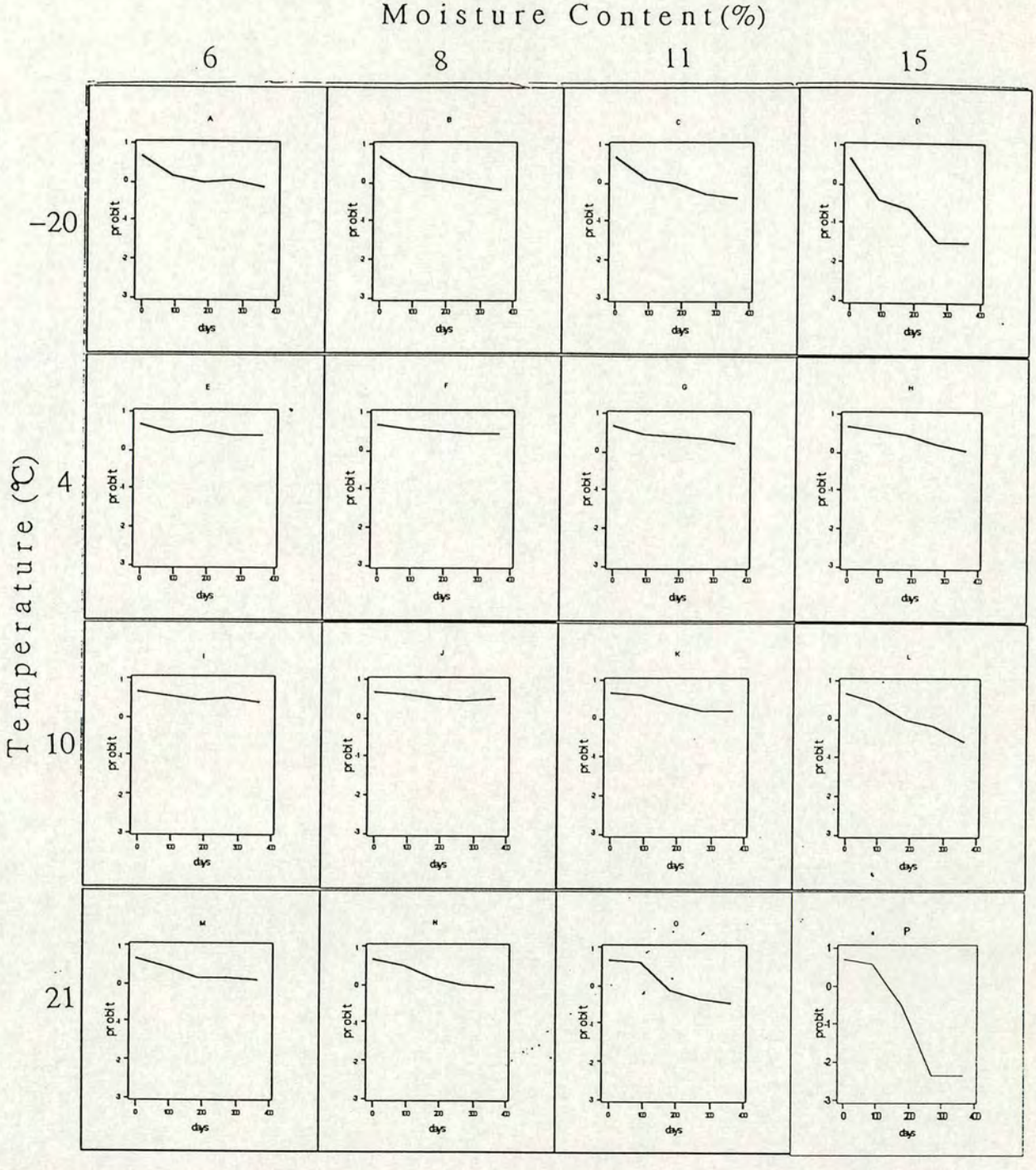


Figure 3.3 Viability of *A. gracilior* seed (from tetrazolium tests, expressed as probit values) against time of storage at specified moisture contents and temperatures. Graphs are organised so that columns correspond to storage moisture content and rows correspond to the temperature during storage.

3.3.7.3 Individual graphs for all factorial combinations of moisture content and temperature

Individual graphs for all combinations of moisture content (columns) and temperature (rows) are presented in Figure 3.3. Comparing the columns, viability is generally low in rows 1 and 4, with temperatures of -20 and 21 °C respectively. All columns (except 4) show relatively high viability maintained in rows 2 and 3, with temperatures of 4 and 10 °C respectively.

On the other hand, the trend of viability across the rows is more regular. Viability decreases with increase of the moisture content in all the rows, particularly at -20 °C and 21 °C. The combination of high moisture content and either of these temperatures results in very poor viability.

3.4 Discussion

The degree of staining of a seed by tetrazolium indicates the level of viability. The interaction of storage moisture content and temperature influences the viability of the seed. The effect of these conditions are explained for each withdrawal.

3.4.1 Timing of effects of moisture contents and temperatures on tetrazolium staining

In the first withdrawal (storage period of 90 days), it can be seen from Table 3.1a that all moisture contents combined with a temperature of -20 °C showed relatively low viability. This indicates that subzero temperatures reduce viability within a relatively short period of time, possibly due to the formation of ice crystals which could kill the seed. The damage on the seed was particularly severe when this freezing temperature of -20 °C was combined with the moisture content of 15%: this could agree with the report by Enescu (1981) that for some species, there was a large range in the abnormal tetrazolium counts after the seeds had been prechilled.

In the second withdrawal (after 180 days) the seeds stored at -20, 4 and 10 °C had a similar staining type to that of the first withdrawal. However, those seeds stored at 21 °C were found to be poorly stained. This significant reduction of viability

by the second withdrawal was probably due to the increased metabolic rate of the seeds which could germinate in storage if sufficient moisture were available but died because this was not the case. The interaction of the higher moisture content (15%) with the 21 °C or even with 10 °C appeared to have increased the rate of seed deterioration, which could be due to the encouragement of early stages of germination followed by seed death because of desiccation intolerance. This point is in agreement with the report by Berjak *et al.* (1990) that even under the most severely desiccating conditions that can be imposed on the whole seed, some germination-associated metabolism, and concomitantly, increasing desiccation sensitivity, may occur. The duration of germination-associated events and subsequent seed death depends on the species and the combination of the storage conditions. For example, the viability of *A. gracilior* declined after 180 days in storage at 15% moisture content and 21 °C (Table 3.1b). At temperatures of 4 or 10 °C combined with any of the moisture contents (with the exception of 15% moisture content combined with a temperature of 10 °C) seed viability was not significantly different from that of their corresponding combinations in the first withdrawal. This indicated that seed deterioration increased with an increase in moisture content and temperature.

In the third withdrawal (after 270 days storage), viability continued to decline but in general, not very significantly from the second withdrawal. However, the seed stored at 21 or -20 °C each in combination with the moisture content of 15% showed a rapid decline in viability (less than 1%, for example, at 15% moisture content and 21 °C). This indicates that loss of viability is not only a function of storage conditions but also a function of time. For example, it has been found that in seeds with higher moisture content the percentage of chromosome aberration and seed damage increased sharply with time (Villiers and Edgcumbe, 1975). They pointed out that an increasing number of abnormalities was observed with increase in time of storage, including distorted cotyledons with necrotic patches of tissues and necrosis of the radicles. This observation agrees with the uneven staining of *A. gracilior* seeds with tetrazolium found in both the gametophyte and the embryo.

By the fourth withdrawal (after 365 days storage) there was not only a complete loss of viability of seed stored at 15% moisture content and 21 °C, but also a significant reduction in viability of seed stored at moisture contents of 11, 8 or 6%

combined with a temperature of 21 °C. Thus for seed to be kept for at least one year without a significant loss in viability the optimal storage conditions should be a moisture content of 6 or 8% combined with a temperature of 4 or 10 °C. At these temperatures seed viability was maintained at an intermediate level with 11% moisture content, and also with 15% moisture content at 4 °C. In this latter case it appears that the metabolic activity was low (due to the low temperature) and it took at least one year before about 50% of the seeds became sensitive to desiccation (Table 3.1d).

3.4.2 Interpretation of staining

The pattern of seed staining by tetrazolium varies widely between species. In *A. gracilior*, good quality seed is clearly stained pinkish-red to dark-red while completely dead seeds show no staining at all. In this experiment a certain proportion of both the gametophyte and embryo in all withdrawals showed a patchy staining with tetrazolium. This was interpreted as those seeds in which viability was beginning to fall. Some researchers interpret the staining pattern for a viable seed to be only those seeds which are completely stained. For example, Enescu (1981) pointed out that for weaker seeds, test results are more variable; the tetrazolium method can be acceptable, provided that only seeds which are completely stained are considered viable. It was observed from this test that the lighter the staining, the less viable the seed was. Interpretation requires experience, skill and good judgement as the result of the test can only be viable or not viable. One has to know the critical areas in the embryo which determine viability. Overaa (1979) evaluated the embryo for tetrazolium tests as follows: radicle area - critical zone; cotyledon area - non critical zone. An embryo is considered viable if:

1. it is completely stained;
2. not more than 1/3 of the root (conical tip) is unstained. This conical tip is not in the critical area, where only small superficial unstained spots can be allowed: outside the critical zone up to 50% of the cotyledon can be unstained or missing.

3.4.3 Value and applicability of the test

In this study it was found that the tetrazolium test can be easily applied to *A. gracilior* seed: there were no practical or technical problems involved in carrying out the test in the laboratory. Some of the reasons for applicability of the test to this species are:

1. seed of *A. gracilior* does not cause the tetrazolium solution to become turbid so that observation is relatively straightforward;
2. *A. gracilior* can give best staining (dark-red), less staining (light-red), patchy staining (uneven staining) or no staining at all so that the tested seeds can be easily categorised;
3. microorganisms were not found to have grown on the seed of this species during the staining period of 24-48 hours.
4. the tetrazolium test can be carried out at room temperature, compared to incubation at 30 °C for a germination test.

3.5 Conclusions and Recommendations

1. Tetrazolium method appears to overestimate viability compared to the germination method. However, trends of declining viability lines of the two methods correlate positively.
2. The best storage conditions are the moisture contents of 8 and 6% interacted with the temperature of 10 or 4 °C.
3. In future work, it is feasible that with good experience and interpretation of the staining pattern, the results of a corresponding germination can be estimated much closer to the actual germination values.

CHAPTER 4

Preliminary experiment on storage of *Afrocarpus gracilior* seed

4.1 Introduction

One of the most basic tools for forest conservation is the temperature-controlled seed store. Forest tree seeds can be stored in optimum conditions for a long time to be used later for a reforestation programme. However, not all seeds can be safely stored without a proportional loss of viability: the rate of seed ageing is dependent on storage environment over time (Ellis and Roberts, 1981). Seeds of *orthodox* species need to be dried (e.g. close to 5% moisture content) while *recalcitrant* species need more moisture (above 20%) for survival. In this experiment, an attempt was made to investigate the optimum storage condition for *A. gracilior* seed. It was a preliminary study carried out over 12 months, the experimental design being a factorial combination of moisture content and temperature. Moisture contents ranged between 6 and 15%, assuming that the seed could experience both extremes in natural conditions. The lower limit might occur in a very dry season after seed falls from a mother tree; 15% moisture content might occur either when seed dries or when it gets into moist soil. It is known that seeds may lie in the soil without germinating for very long periods (Villiers and Edgcumbe, 1975).

The purpose of this experiment was to investigate the optimum storage conditions for *A. gracilior* seeds to prolong viability measured by germination method.

4.2 Materials and Methods

This experiment was set up to run in parallel with the tetrazolium chloride experiment for *Afrocarpus gracilior* (A2 seedlot) described in Chapter 3. The seed origin, experimental design and methodology for setting the seed moisture content and storage temperature up to withdrawal and seed splitting are identical to those in

Chapter 3 but the number of seeds used in this experiment was twice those used in Chapter 3 (tetrazolium chloride test). For each of the 16 treatments (4 moisture contents × 4 storage temperatures) 120 seeds were sealed in 16 × 20 cm aluminium bags and withdrawn after periods of 90, 180, 270 and 365 days. This enabled 4 replications of 25 seeds each to be prepared for the germination test, taking account of some seeds being damaged (and discarded) in the process of seed coat removal by a vice. The naked seeds were sown into 0.5 - 1.2 mm quartz sand from the Edinburgh area, which was sterilised by heating to 150 °C and cooling down to ambient temperature. One hundred seeds were randomly taken from the 120 (or less depending on how many were discarded because of damage during splitting) and sown about 1 cm deep in the sand.

In the first withdrawal, all 100 seeds were sown in aluminium trays of 36 × 28 × 6 cm covered with cling film. In this test, replications were done by demarcating the sand into four blocks, 25 seeds sown in each block. Regarding the mixture of water and sand, 45 ml of water was used for 1 kg of sand in the first and second withdrawals: this was reduced to 15 ml water to 1 kg sand in the third withdrawal. Plastic food boxes were used in the second and third withdrawals with a tightly fitting cover in which 25 seeds were sown and replicated four times. By the fourth withdrawal, it became apparent that the low germination results must be due to inappropriate materials and methodology. Hence, most of the materials and methods were changed. In the last withdrawal Garside Industrial sand was used, the ratio of water to sand was 15 ml water to 1 kg of sand (as in the third withdrawal), plastic trays with 14 holes in the bottom were used with 25 seeds sown in each tray and replicated four times (i.e. separate trays for each replication) and each tray was put in a plastic bag with the open end folded twice and clipped at one point in the middle to allow some ventilation.

The trays were randomly placed in the growth room at 30 °C with the relative humidity, 45%. The experiment was assessed for germination every 15 days, up to 6 weeks. Germination was defined as emergence of the radicle to 10 mm. Germinated seeds were recorded and removed from the trays.

In conclusion of materials and methods, it should be noted that the control treatment (11% MC and 10 °C) stored soon after seed collection in Ethiopia had altogether six withdrawals for viability determination, the second being equivalent to the 'zero withdrawal' at the start of this experiment. Hence, the statistical basis for correcting data for the effect of using inappropriate media (discussed in Section 4.3.5) was based on the six withdrawals of the control.

4.3 Results

Mean viability data are summarised in Table 4.1 and Analyses of Variance are presented in Table 4.2. (These data have been adjusted as mentioned below.) The ranking of viability means is detailed in Appendix 4.1. The effects of moisture content, storage temperature and their interaction on germination at each withdrawal are described in Sections 4.3.1 to 4.3.4. Section 4.3.5 deals with adjustment of data to compensate for the use of inappropriate media and techniques in the first three withdrawals. Section 4.3.6 describes the introduction of 'zero time' withdrawal data, and the trends of decreasing viability with time are discussed in Section 4.3.7. Percentage data were transformed into probit values, as mentioned in Chapter 3, to compare the mean slopes of decreasing viability lines over time for different storage treatments.

4.3.1 First withdrawal (Table 4.1a)

Mean viability percentages for 6, 8, 11 and 15% moisture content were 23, 22, 20 and 32% respectively and analysis of variance indicated that the moisture content effect was barely significant ($F = 2.63$, $P = 0.06$, Table 4.2). Temperature was relatively more important at this withdrawal: mean viability percentages at -20, 4, 10 and 21 °C were 13, 32, 29 and 24% respectively and the effect of temperature was highly significant ($F = 16.92$, $P < 0.001$). The interaction effect was also highly significant ($F = 8.12$, $P < 0.001$). The highest germination occurred when 15% moisture content combined with 4 °C, and every moisture content treatment combined with a temperature of -20 °C produced a poor result.

Least significant differences (LSD) were calculated using the standard formula, $2\sqrt{2 \times \text{Error mean square}}$. Mean viability (in Probit scale) was ranked by using LSD and the superscripts in Table 4.1 indicate whether the difference between any two individual means was significant or not. Any overlapping of the ranking letters shows that there was no significant difference between the specified means.

Based on the calculated LSD, there was no significant difference between mean viability for the 4 moisture contents other than between 11 and 15%. Viability at -20 and 21 °C were significantly different from each other and also from that at 4 and 10 °C. The combination of 15% moisture content and a temperature of 4 °C is significantly different from most of the other combinations.

4.3.2 Second withdrawal (Table 4.1b)

Mean viability percentages for 6, 8, 11 and 15% moisture-contents were 16, 12, 14 and 11% respectively and analysis of variance for the moisture content was highly significant ($F = 16.24$, $P < 0.001$, Table 4.2). Comparison of F values indicates that the temperature effect was again more important than the moisture content effect although their probability levels were the same.

Percentage viability for the temperatures of -20, 4, 10 and 21 °C were 7, 22, 15 and 9% respectively: viability at 4 °C was significantly different from all other temperatures and at 10 °C was significantly higher than -20 and 21 °C.

The interaction remained highly significant, with little change in F value from withdrawal 1. The combination of 15% moisture content and 4 °C maintained relatively high viability but was not significantly different from other combinations with 4 °C. In contrast to withdrawal 1, mean viability at 15% was significantly lower than for other moisture contents.

(a) WD1

Temperature °C	Moisture content % 6	8	11	15	$\bar{X}$
-20	-0.10^{fghijk}	0.00^{efghij}	-0.15^{ghijkl}	-0.87^{no}	-0.28^{d}
4	0.32^{cdef}	0.35^{bcde}	0.14^{cdefgh}	0.81^{a}	0.41^{a}
10	$0.09^{cdefghi}$	0.26^{cdefg}	0.50^{abc}	0.44^{abcd}	0.32^{a}
21	0.35^{bcde}	-0.21^{hijklm}	-0.50^{klmn}	0.76^{ab}	0.10^{c}
$\bar{X}$	0.17^{ab}	0.10^{abc}	-0.00^{bcd}	0.29^{a}	

(b) WD2

Temperature °C	Moisture content % 6	8	11	15	$\bar{X}$
-20	-0.14^{bcdefg}	-0.52^{ijklm}	-0.49^{hijkl}	-1.19^{n}	-0.58^{cd}
4	0.09^{ab}	0.08^{abc}	0.01^{abcd}	0.29^{a}	0.12^{a}
10	0.00^{bcde}	-0.44^{hijk}	0.01^{abcd}	-0.07^{bcdef}	-0.12^{b}
21	0.28^{efghij}	-0.23^{defgh}	-0.24^{defghi}	-1.19^{n}	-0.48^{c}
$\bar{X}$	-0.08^{a}	-0.28^{bc}	-0.17^{ab}	-0.54	

(c) WD3

Temperature °C	Moisture content % 6	8	11	15	$\bar{X}$
-20	-0.37^{defg}	-0.42^{ghij}	-0.44^{ghijk}	-1.19^{lm}	-0.60^{d}
4	0.14^{ab}	0.21^{a}	0.08^{abc}	0.08^{abc}	0.13^{a}
10	-0.02^{bcdef}	-0.02^{bcdef}	0.00^{bcde}	-0.24^{g}	-0.07^{b}
21	0.05^{abcd}	0.00^{bcde}	-0.27^{gh}	-0.06^{l}	-0.32^{c}
$\bar{X}$	-0.08^{a}	-0.06^{ab}	-0.17^{ab}	-0.60	

(d) WD4

Temperature °C	Moisture content % 6	8	11	15	$\bar{X}$
-20	-0.29^{defg}	-0.43^{ghij}	-0.37^{fgh}	-2.07^{m}	-0.79^{c}
4	-0.20^{bcdef}	-0.10^{abc}	-0.20^{bcdef}	-0.12^{abcd}	0.15^{a}
10	-0.02^{a}	-0.15^{abcde}	-0.38^{ghi}	-0.91^{l}	-0.36^{b}
21	-0.37^{fgh}	-0.07^{ab}	-0.71^{k}	-2.07^{m}	-0.81^{cd}
$\bar{X}$	-0.22^{ab}	-0.19^{a}	-0.29^{bc}	-0.41^{d}	

Table 4.1 Mean viability (probit scale) of *A. gracilior* seed from germination tests: The significance of a difference between any two means (within any single withdrawal, as each withdrawal was analysed separately) was tested by the LSD test; means with different superscripts (a,b,c, ...) are those that have been declared significantly different from each other (using probit transformation). The column and row *means* were considered separately.

Analysis of Variance for WD1

Source	DF	SS	MS	F	P
MC	3	0.69347	0.23116	2.63	0.061
Temp	3	4.46814	1.48938	16.92	<0.001
MC*Temp	9	6.43301	0.71478	8.12	<0.001
Error	48	4.22472	0.08802		
Total	63	15.81935			

Analysis of Variance for WD2

Source	DF	SS	MS	F	P
MC	3	1.88336	0.62779	16.24	<0.001
Temp	3	5.04632	1.68211	43.52	<0.001
MC*Temp	9	3.75364	0.41707	10.79	<0.001
Error	48	1.85522	0.03865		
Total	63	12.53854			

Analysis of Variance for WD3

Source	DF	SS	MS	F	P
MC	3	3.28786	1.09595	54.62	<0.001
Temp	3	4.79442	1.59814	79.64	<0.001
MC*Temp	9	1.89958	0.21106	10.52	<0.001
Error	48	0.96319	0.02007		
Total	63	10.94505			

Analysis of Variance for WD4

Source	DF	SS	MS	F	P
MC	3	12.9310	4.3103	309.89	<0.001
Temp	3	5.0345	1.6782	120.65	<0.001
MC*Temp	9	7.0291	0.7810	56.15	<0.001
Error	48	0.6676	0.0139		
Total	63	25.6622			

Table 4.2 Analyses of variance for germination tests, withdrawals (WD) 1, 2, 3 and 4.

4.3.3 Third withdrawal (Table 4.1c)

Percentage viability for moisture contents of 6 and 8% were higher than for 11 and 15%. Mean viability percentages for 6, 8, 11 and 15% moisture content were 17, 17, 14 and 8% respectively. Analysis of variance showed that moisture content remained highly significant, although the F value continued to increase.

Percentage viability for temperatures of -20, 4, 10 and 21 °C were 6, 22, 16 and 12% respectively. As in withdrawal 2, the highly significant temperature effect remained greater than that of moisture content.

Analysis of variance showed the interaction was more or less the same as that for withdrawals 1 and 2. A temperature of 4 °C combined with all moisture contents resulted in higher viability percentage than other combinations of moisture content and temperature (Table 4.4c).

4.3.4 Fourth withdrawal (Table 4.1d)

Analysis of variance indicated that the moisture content effect was highly significant ($F = 309.89$, $P < 0.001$) with the F value greater than that for temperature. The mean viability percentages for the moisture contents of 6, 8, 11 and 15% were 41, 43, 34 and 16% respectively. The percentage viability at 15% was significantly lower than at other moisture contents.

For temperature, mean viability percentages for -20, 4, 10 and 21 °C were 27, 44, 36 and 26% respectively. Analysis of variance showed that the temperature effect continued to be highly significant ($F = 120.65$, $P < 0.001$). Viability at 4 °C was significantly higher than at other temperatures: the highest individual viability was at 10 °C in combination with 6% moisture content, which was not significantly different from 10 °C at 8%.

Analysis of variance showed that the interaction effect for the fourth withdrawal was approximately five times higher than that of the three previous withdrawals (Table 4.2).

Broadly speaking, every moisture content in combination with 4 °C and the moisture contents of 6 and 8% in combination with 10 °C gave a relatively good germination.

4.3.5 Adjustment of results for medium effect

The results of the first withdrawal were significantly lower than the viability of the control on arrival, as tested at Alice Holt (Figure 4.1c) and because of this the question was asked whether such a loss in viability was normal or whether the materials and methods used for germination were inappropriate. Three factors that might affect viability were considered - the ratio of water to sand, the aeration of the containers, and the type of sand (the initial assessments at Alice Holt were carried out using Garside sand). Side experiments were not possible because of limited seed supply and the alternative adopted was to modify germination methodology. In the second withdrawal the trays were changed, in order to ensure replication, to food boxes (without holes in the bottom). In the third withdrawal the ratio of water to sand was reduced and in the fourth withdrawal the type of sand was changed to that used in the initial germination test prior to the experiment (Garside) and the food boxes were replaced by trays with holes. It was not until the fourth withdrawal, where results showed an increase in viability, that the main reasons for poor germination mentioned above seemed clear. It was then decided to adjust the germination percentages for withdrawals 1, 2 and 3 on the basis of data for the control treatment (11% moisture content).

The control treatment was considered as an extension of the seed which had been stored in Addis Ababa at a moisture content of 11% and temperature of 10 °C. There were six germination tests carried out on this seed, the first two at Alice Holt when the seed was 175 and 295 days old respectively from the date of collection. The second test coincides with zero withdrawal for the actual storage experiment.

germination test	storage withdrawal
1	(pre storage)
2	(zero)
3	1
4	2
5	3
6	4

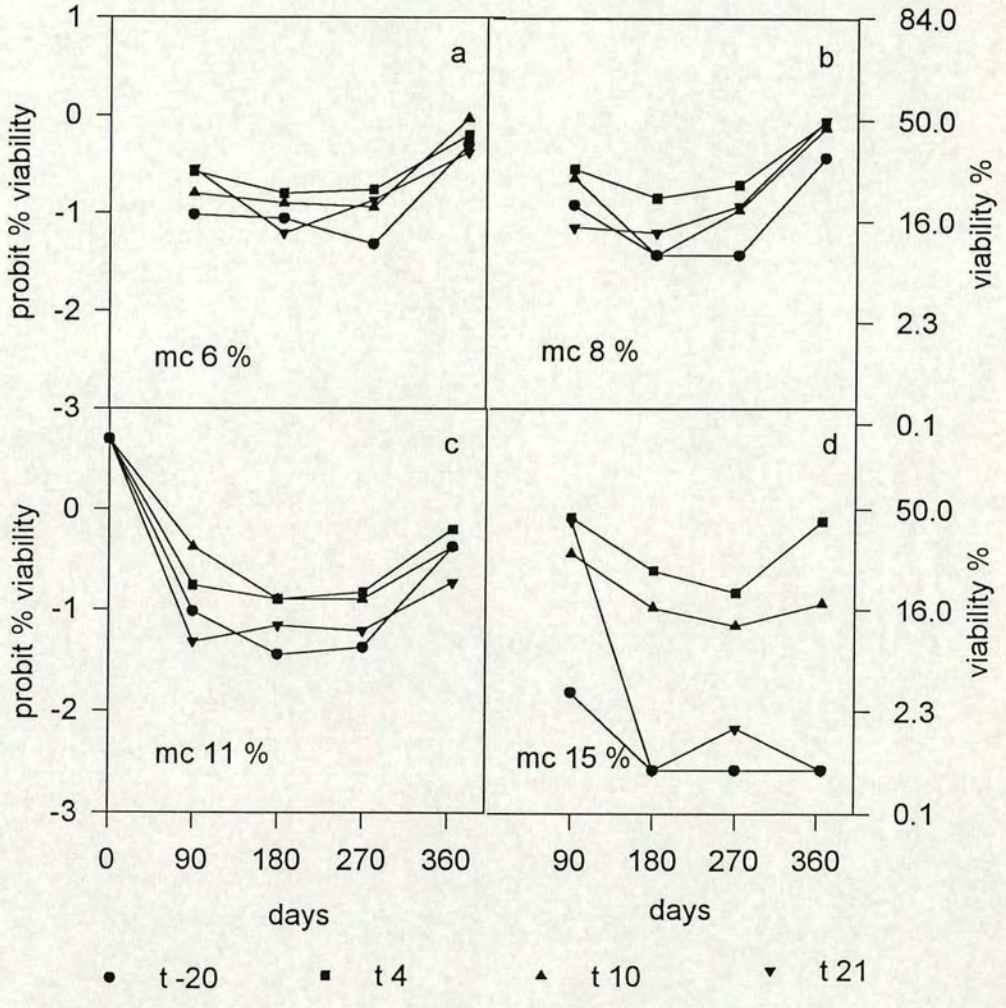


Figure 4.1 A2 shows viability in the factorial interaction of the moisture contents of: a. 6, b. 8, c. 11 and d. 15% with the temperatures of -20, 4, 10 and 21 degrees. C is a control and each factorial was considered to have a viability measured at zero time.

There were two reasons for considering the control treatment to estimate the effect of media:

(a) the control seed was not subjected to any enforced desiccation or rehydration;

(b) the control seed had viability tests before zero storage, at zero storage and subsequently during the storage period.

The first three withdrawal tests in the storage period were done under different germination methodology which resulted in a medium effect as mentioned above. The effect was removed by dividing the control data into two for Medium 1, (tests 1, 2 and 6) and Medium 2, (tests 3, 4 and 5). The media are described as follows:

Medium 1: Water to sand ratio 15 ml water to 1 kg of sand, germination tray had 14 holes, Garside Industrial sand was used.

Medium 2: Water to sand ratio 45 ml water to 1 kg of sand, germination box without holes at the bottom, Edinburgh local sand used.

Two parallel lines were fitted to data points for Medium 1 and 2 (Figure 4.2a): percentage results were transformed into probit values and the mean for each medium was calculated, 0.273 and -0.612 for Medium 1 and 2 respectively. The difference between Medium 1 and Medium 2 (0.885 probit units) was added to the probit values of Medium 2 for all treatments in withdrawals 1, 2 and 3. This is shown Figure 4.2b where the probit values of Medium 2 have moved in line with Medium 1.

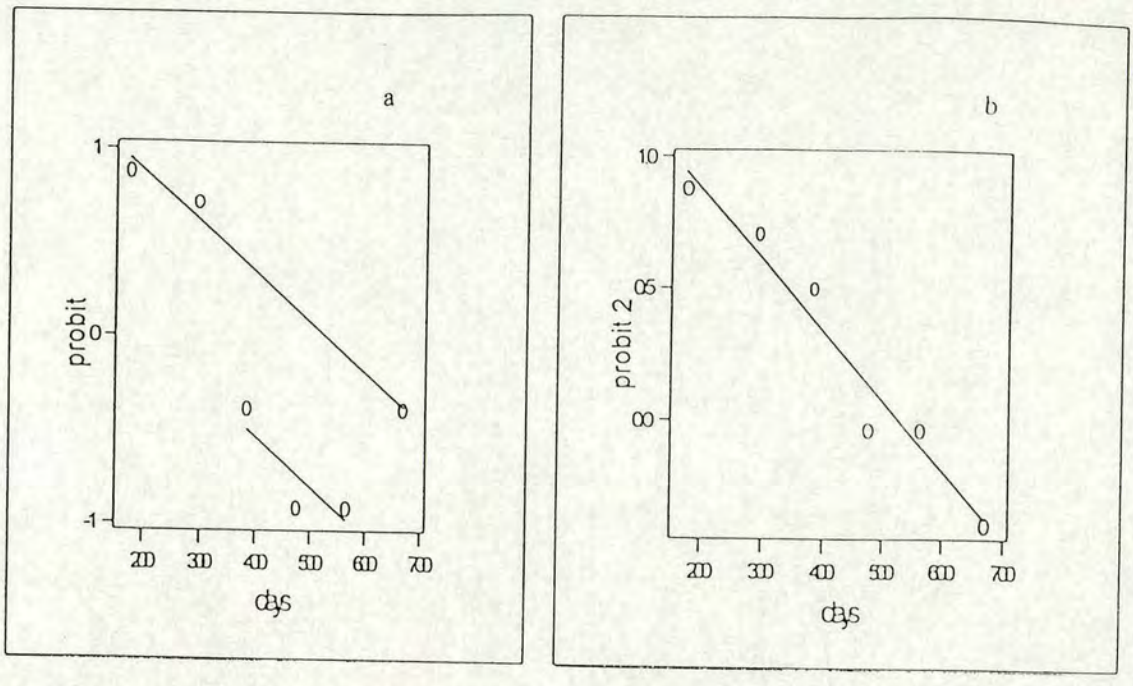


Figure 4.2 Adjustment of germination percentage (probit scale) (a) before adjustment: upper line represents Medium 1; lower line represents Medium 2, (b) after adjustment.

4.3.6 Gradient of decreasing viability lines

'Zero withdrawal' tests were not carried out immediately after drying (MC 6 and MC 8%) and rehydration (MC 15%) but only the control seed (MC 11%) was tested when the storage began. Analysis of variance calculated for the slopes of decreasing viability lines without 'zero' withdrawal points showed moisture content to be highly significant ($F = 68$, $P < 0.001$), temperature less so ($F = 3.88$, $P < 0.05$) and their interaction also highly significant ($F = 11.02$, $P < 0.001$).

This is similar to analysis of variance of slopes including zero withdrawal points (Table 4.3, ranking of slopes is shown in Appendix 4.2). However all F-values without 'zero' withdrawal points were less than those including 'zero' withdrawals. Moreover, F-value for the temperature without 'zero' withdrawal was significant only at 0.05 probability. The trends of decreasing viability lines including 'zero' withdrawal points are now considered in detail.

Analysis of Variance (Balanced Designs)

Analysis of Variance for b(WD0-4)

Source	DF	SS	MS	F	P
MC	3	112.202	37.401	209.54	0.001
Temp	3	26.956	8.985	50.34	0.001
MC*Temp	9	51.134	5.682	31.83	0.001
Error	48	8.567	0.178		
Total	63	198.859			

MEANS

	Moisture content %				
Temperature °C	6	8	11	15	$\bar{X}$
-20	-2.48^{cdefg}	$-2.94^{ghijklm}$	-2.68^{efghi}	-6.45^{o}	-3.63^{c}
4	-2.19^{bcdef}	-1.92^{abc}	-2.04^{abcd}	-2.62^{defgh}	-2.19^{a}
10	-1.72^{ab}	-2.17^{bcde}	-2.92^{ghijkl}	-4.30^{n}	-2.78^{b}
21	-2.70^{efghij}	-1.47^{a}	-2.87^{ghijk}	-8.11^{p}	-3.78^{cd}
$\bar{X}$	-2.27^{ab}	-2.12^{a}	-2.63^{bcd}	-5.37^{d}	

Table 4.3 Analysis of variance and means for the slopes of decreasing viability lines for withdrawals 0 to 4. Mean slopes are ranked by LSD: values with the same superscript are not significantly different.

4.3.7 Trend of viability (adjusted for medium effect and including zero withdrawal)

The viability trends for withdrawals 0 to 4 are shown grouped in Figures 4.3 and 4.4 for moisture contents and temperatures respectively, and individual lines for each combination of storage conditions are presented in Figure 4.5. Detailed comments on each graph are not appropriate in this preliminary experiment because the original germination data were adjusted for the 'medium effect'. For example, viability appears to increase in withdrawal 1 for 15% moisture content combined with 4° and 21 °C. Results are therefore limited to main effects and trends.

4.3.7.1 Effect of moisture content (Figure 4.3)

There was a noticeable decrease in viability in the first 90 days. The decrease from 90 to 365 days is generally linear but at 15% moisture content combined with -20° and 21 °C the slope is steep and irregular. There is little variation in slope between temperatures at each moisture content apart from the 15% moisture content already mentioned.

4.3.7.2 Effect of temperature (Figure 4.4)

The decrease in viability is linear at 4° and 10 °C but at both -20° and 21 °C there was little decrease after 180 days storage, apart from 15% moisture content. At 4 °C there was no significant difference in slope between moisture contents.

*4.3.7.3 Trends of moisture content*temperature combinations (Figure 4.5)*

When all results are placed together it is possible to discern the overall pattern, and to select the optimal conditions for storage. It seems that germination rate is best when the storage temperature is 4 °C, at which temperature the moisture content (at least within the experimental range) was not very important.

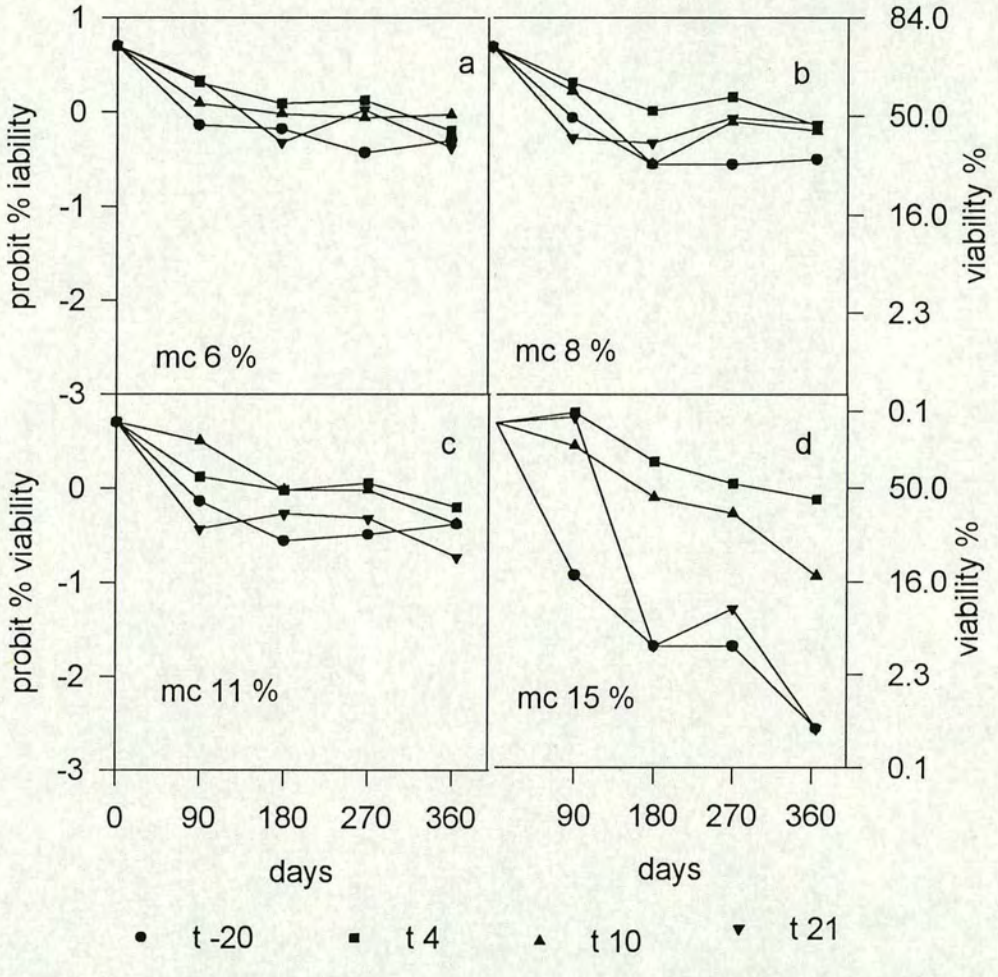


Figure 4.3 Viability trends for each moisture content.

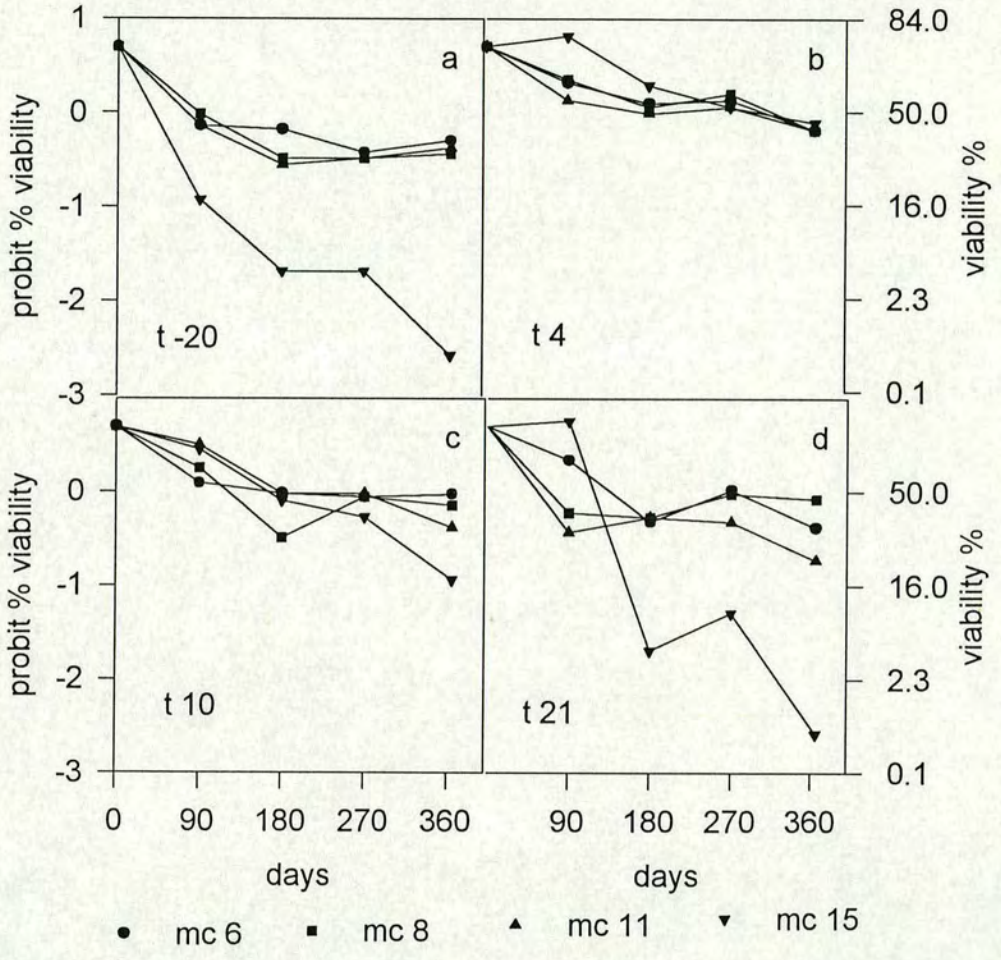


Figure 4.4 Viability trends for each temperature

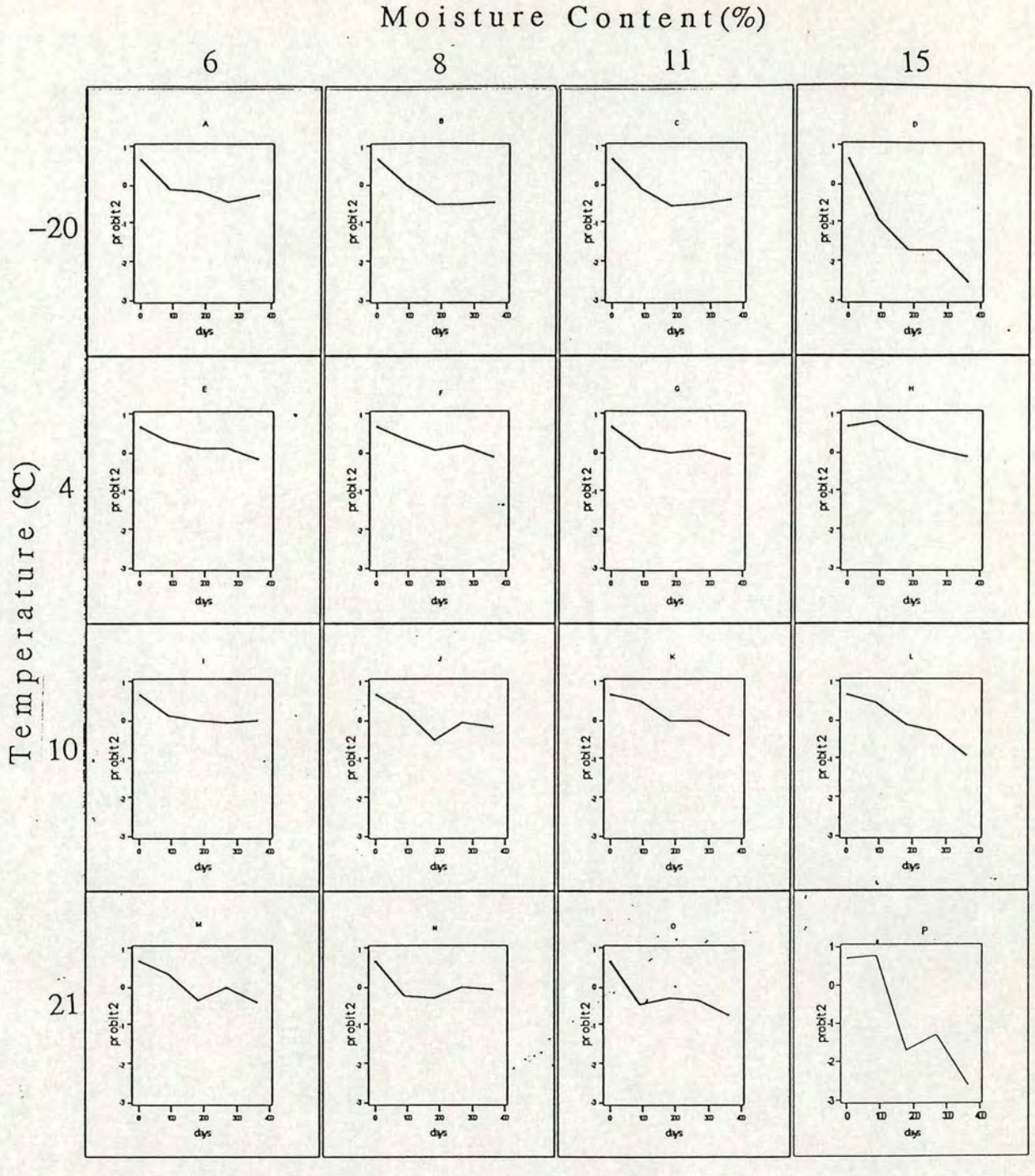


Figure 4.5 Compilation of complete data set on the effect of storage moisture content, tempeature and time on the germination of *Afrocarpus gracilior* seeds. Germination rate is expressed as the probit transformation.

4.4 Discussion

4.4.1 Seed storage category of *A. gracilior*

Results of the experiment suggested that the seeds of *A. gracilior* tolerated desiccation to around 6 to 10% moisture content (wet basis). However, this was not proved since viability tests were not carried out immediately after desiccation treatment. Despite this, it was observed from subsequent withdrawals that viability at 6 and 8% was better than at higher moisture contents. Storage at sub-zero temperatures of -20 °C caused considerable damage to the seed. This result is not in agreement with either recalcitrant or orthodox seed storage behaviour. However, it is similar to results obtained by Ellis *et al.* (1991b) on papaya (*Carica papaya*), whereby the seed tolerated desiccation to between 7.6 - 9.4% moisture content, which lost viability faster in cooler conditions. This behaviour would classify *A. gracilior* into intermediate seed storage category.

Bass (1975) in Hong and Ellis (1995) confirmed that papaya can be stored at moisture contents in equilibrium with 50% r.h. (9-10% moisture content) and 10 °C. Hence, both the calculated storage conditions and the comparisons with other intermediate species, e.g. coffee, which thrive in the same ecological niche with *A. gracilior* could suggest that *A. gracilior* should be classified as intermediate seed storage category. Regarding the temperature for the storage condition for *A. gracilior*, it has been found to be between 4 and 10 °C but appears slightly close to 10 °C (see Table 4.3). A further example of intermediate seed reported by Hong and Ellis (1995) has been *Citrus* spp. with the storage moisture content which could agree with the result obtained for *A. gracilior*.

4.4.2 Effect of storage conditions on seed viability

The reduction in viability during the first 90 days is due to desiccation intolerance caused by two factors:

(i) reduction of moisture content (to 6 and 8%) after 260 days stored in Addis Ababa at 11% and 10 °C, and 35 days in Edinburgh at approximately 20 °C;

(ii) the sub-ambient temperature of 10 °C in Addis Ababa may have broken dormancy and exacerbated the above desiccation damage. The fact that re-hydrated seeds gave higher germination in the first withdrawal supports the view that storage in Addis Ababa might break dormancy and the seeds might be on sub-lethal damage which could be repaired.

The drop in viability during the first 90 days for seed maintained at 11% moisture (as during storage in Addis Ababa) is probably due to the higher temperature experienced in Edinburgh (prior to the experiment) which would start pre-germinative processes requiring more moisture.

According to Ellis *et al.* (1991a) *Coffea arabica*, in the intermediate seed storage category, was more vulnerable to desiccation sensitivity than orthodox seeds at the initiation stage of germination. Premature germination might be initiated at any time before storage (during seed processing for example). In coffee seed, germination has been initiated during the course of maturation and proceeds throughout imbibition: all treatments in which germination would be expected to have been initiated, or to have proceeded further, increased desiccation sensitivity (Ellis *et al.*, 1991a).

Koster and Leopold (1988) reported that both the emergence and leakage studies on orthodox seed confirm that desiccation tolerance of the radicle is lost soon after the emergence from the seed coat. Hence, *A. gracilior* could be grouped with coffee in that desiccation sensitivity (lethal point of water) is reached before the radicle emerges. In *A. gracilior* variance due to moisture content was lower than that of temperature until the third withdrawal, after which variance due to moisture content overtook that of temperature (Table 4.2). This was the point where the difference between moisture treatment became high. It might be that seeds of most moisture treatments became desiccation sensitive and that probably seed death had taken place (e.g. those at 15% MC) after the third withdrawal.

Another reason for an effective decrease of viability between withdrawal 0 and withdrawal 1 (90 days) for the moisture contents of (6, 8, and 11%) (see Figures 4.4 and 4.5) was that it was a common practice in Ethiopia to soak fresh seeds of *A. gracilior* for about three days so as to remove the fleshy coverings. There is evidence to support the idea that seeds would lose their desiccation tolerance if they are subjected to pre-soaking for some hours. Pre-soaking, even for as short a period as three hours, has deleterious effects on the ultrastructure of rye (*Secale cereale*) embryos when dehydrated to their initial moisture content, Sargent *et al.* (1981) in (Hong and Ellis, 1995). Similarly, Hong and Ellis (1992b) reported that mung bean which had been imbibed for eight hours lost 5% viability when dried back to between 4.3% and 6.4% moisture content, despite no seeds germinating during this imbibition treatment. The pre-soaking of *A. gracilior* seed for about 3 days might have started precocious (premature) germination.

Yet another reason for the early decrease in viability could be related to the fact that *A. gracilior* gametophyte-embryo is quite oily compared to the seed coat. On the contrary the moisture content of the seed coat is about 2.5 times higher than the moisture content of the gametophyte plus embryo. Thus the moisture content of the seed is mainly that of the seed coat. This would mean that the moisture content of the gametophyte plus embryo might not be properly estimated as its moisture content is overshadowed by the moisture content of the seed coat. What actually determines the right level of moisture content for survival will be the moisture content of the gametophyte and particularly of the embryo. When this is more than the optimum, it may result in premature germination. Further dehydration of such seeds can inflict damage on the seeds because of desiccation intolerance of the seeds. There is evidence that oily seeds should be dried further down to have an equivalent water potential with that of the starchy seeds. This shows that the oily gametophyte plus embryo has a higher water potential than the seed coat. If the seed is stored before the gametophyte plus embryo moisture content comes in equilibrium with that of the seed coat, viability can be lost particularly on further desiccation of the seed.

4.4.3 Storage treatment for primed and dried seed

Seed rehydrated to 15% moisture content ('primed') showed that viability can be maintained provided storage temperature is sub-ambient but not sub-zero. In the experiment, 4 °C and to a less extent 10 °C achieved this, but both 21° and -20 °C resulted in rapid fall in viability.

The alternative dried seed retains viability because of its low level of metabolic activity. The results showed that storage at 6 or 8% moisture - at a wide range of temperature, excepting sub-zero - maintained viability equally well to the primed seed above.

Both groups of seeds (primed and dried) have survived because both have met different physiological requirements which enabled them to survive. According to Heydecker (1977), dry seeds survive because their metabolic processes are greatly slowed down, whereas imbibed dormant seeds, though not germinating, are nevertheless highly active in other respects and continually repair any cytological damage they have experienced; they thus remain at the peak of their performance capacity for much longer than if they were dry. The seed under environmental stress can not germinate. At some stage along the scale of moisture content for example, water stress can delay germination for a long or short time. Heydecker (1977) reported that when re-imbibition is arrested somewhere between the unimbibed quiescent and fully imbibed germinative condition the embryo is caught in a stress situation in which it can be maintained for days or weeks. In the case of this experiment, 11% moisture content appears to be in the upper limit of the dried quiescent stage, i.e. intermediate between the dried and primed condition.

4.4.4 Effect of germination medium on viability

Other reasons for the low germinability of the seeds at withdrawals 1 (90 days), 2 (180 days) and 3 (270 days) was the type of sand used for germination. It was observed that the Edinburgh local sand used for germination was clayey and in some cases formed suspension when the water ratio was increased but became compact when the ratio of water to sand was reduced. This condition appeared to hamper air

circulation and might have also physically hindered the emergence of germinating embryo.

Another important factor of germination methodology which occurred particularly at withdrawal 2 (180 days) was the change of germination tray to food boxes. In the first withdrawal, replications were done within the same large tray by dividing the sand into four plots. In the second withdrawal, in an attempt to have replication as individual boxes, four boxes without holes at the bottom were used for each treatment. As the boxes had tight covers the level of oxygen was limited to just what was in the box. This was the point where most seeds showed the least germination, other than a complete loss of viability due to ageing. The absence of good aeration might have resulted in anoxia. According to Heydecker (1977) more oxygen is sometimes required for germination than for growth and in order to trigger germination, oxygen has to be made available for its specific process which differs from conventional 'respiration' and which probably takes the pentose phosphate (PP) pathway. Therefore metabolic processes which inhibit respiration can trigger germination.

The water-sand mixture used in Withdrawals 1, 2 and 3 was different from the one used in the last withdrawal. In the former three withdrawals, the water to sand ratio was 45 ml to 1 kg of sand. As it was observed later in the last withdrawal, *A. gracilior* appears to be suffocated by excess water in the sand. The water just above requirement seems to affect the normal respiration when germination has started. In the last withdrawal the ratio of water to sand was 15 ml to 1 kg of sand.

For the last withdrawal, the sand type itself was changed. Industrial Garside sand, which the Forestry Commission Southern Research Station used for the same seed before this experiment was started, was used. As a result of several changes in the methodology of germination a better result was achieved in the last withdrawal (Figure 4.1).

4.4.5 Conclusions and Recommendations

The seed storage treatments which best maintained viability of *A. gracilior* in this experiment were as follows:

Moisture content %

Temperature °C	6	8	11	15
-20				
4	√	√	√	√
10	√	√		
21		√		

It is clear that it is best to store seed at 4 °C irrespective of moisture content, and at 10 °C with the moisture contents of 6 and 8%.

CHAPTER 5

Storage experiment of *Afrocarpus gracilior* seed

5.1 Introduction

A seed's longevity is mainly dependent on the moisture content and temperature at which it is stored. Within a species, the seed has an optimum storage condition, although there is likely to be variation between seed lots. This variation could happen for different reasons. Environmental conditions might change from year to year and that could cause some variation between seeds during development on the parent plants. Seed size and vigour are influenced by environmental and genetic factors. According to Ellis and Roberts (1981) the value of K_i (viability immediately after harvest) may differ within a seed lot because it is influenced by preharvest environment and genotypes. Similarly, Pritchard *et al.* (1993) pointed out that temperature is one of a number of environmental factors that influence seed development on the parent plant with respect to dormancy status which was shown to vary considerably between years and to be associated with average daily temperature maxima and minima during development. The ability of the seed to maintain its viability for a long time depends critically on its initial maturity. Several researchers have directly and indirectly observed that temperature or heat sums, solar radiation and precipitation could lead to seed development, expressed as a maturity index, where the more mature seeds have greater vigour and germination potential (Edwards, 1980). Storage moisture content and temperature limits could also be related to the pre-storage conditions of the seed from its early development on the parent plant. Seed size, seed desiccation rate, seed longevity and the habitat of the parent species have all been found to be related to storage physiology (Tompsett, 1992).

The purpose of this experiment was to investigate the optimum storage condition for *A. gracilior* seeds to prolong viability measured by germination method.

The storage conditions of the present experiment were similar to those of the preliminary experiment on storage of A2 seed (Chapter 4). However, the upper

moisture content treatment was 17% (rehydrated from 10%), and the lower temperature limit was -5 °C. The reason for these changes was that the uppermost moisture content (15%) and the lowest temperature (-20 °C) used in the previous chapter gave the best germination (in the first withdrawal) and the worst respectively. It was then thought that the moisture content might not be high enough in the upper limit and the temperature was too low in the lower limit. Hence, the moisture content and temperature were increased from 15 to 17% and -20 to -5 °C respectively.

5.2 Materials and Methods

The A3 seed lot was collected in March 1994 from Lagalencha, Adaba, Ethiopia, and transported to Wondo Genet (between collection area and Addis Ababa). The seed was soaked in water for two days and the pulp removed by hand and discarded. The seed was dried in the sun for two days and transported to Addis Ababa on a pick-up vehicle. A further drying was done in the shade at the Forestry Research Centre where the seed was stored at just below 10 °C before being transported to Edinburgh by air in the same month of collection. The moisture content on arrival was 10% and the seed was stored at 10 °C in Edinburgh for a period of 40 days before starting this experiment. This seed was about 15% larger in size than A2. Seeds were thoroughly mixed before the storage experiment was established. The design was a factorial experiment of the moisture contents (6, 8, 10 and 17%) and the temperatures (-5, 4, 10 and 21 °C).

The number of seeds taken for the experiment, the method of drying/rehydrating and hermetic storage were exactly the same as for the A2 seed lot (see Chapter 4).

Regarding germination (viability test), the method used in the fourth withdrawal of A2 seed (Chapter 4) was adopted as most appropriate. Thus the germination medium was Garside Industrial sand (15 ml water per kg of the sand), incubated at 30 °C in a growth room of relative humidity 45%, with the trays loosely clipped in polythene bags. Mean viability was calculated in Probit value as in Chapter 4, the means being ranked (a, b, c, etc as a superscript) on the basis of LSD values derived from analysis of variance.

5.3 Results

Mean viability and analyses of variance data for A3 seed are presented in Tables 5.1 and 5.2, and each withdrawal is now described individually. Ranking of mean viability was based on LSD, as shown in Appendix 5.1.

5.3.1 Withdrawal 1

The moisture content had a significant effect on viability ($F = 38.98$, $P < 0.001$). Mean viability decreased with increase of moisture content (Table 5.1a): the differences between moisture contents were significant apart from 6 and 8%.

Viability in relation to the temperature was calculated to be significant ($F = 34.65$, $P < 0.001$). Mean viability was highest at 4 °C although not significantly different from that at 10 °C (Table 5.1a) and decreased at -5 °C and 21 °C.

Viability in relation to the interaction between the moisture content and temperature was not significant ($F = 1.12$, $P > 0.30$). There were no significant differences between the mean viability in relation to moisture contents of 6 and 8% at temperatures of -5, 4 and 10 °C, and similarly, 10% at 4 and 10 °C. The interaction of the moisture content 17% with the temperature of -5, 10 and 21 °C resulted in the lowest seed viability.

5.3.2 Withdrawal 2

The effect of moisture on viability remained highly significant ($F = 100.83$, $P < 0.001$) with viability progressively decreasing as moisture content increased. However, the difference between the means for moisture contents 6 and 8% was again not significant although differences between other moisture contents were significant (see Table 5.1b).

Regarding the effect of temperature, viability at 4 °C was the highest. Viability at -5 and 10 °C was not significantly different and at 21 °C was recorded the least.

The overall interaction of the moisture content and temperature on mean viability was significant ($F = 4.07$, $P = 0.001$). Combinations of moisture contents 6

(a) WD1

Temperature °C	Moisture content %				
	6	8	10	17	$\bar{X}$
-5	60^{abcd}	59^{abcde}	51^{gh}	42^{jkl}	53^{bc}
4	62^{a}	61^{ab}	55^{bcdefg}	49^{ghi}	57^{a}
10	59^{abcde}	60^{abc}	58^{abcdef}	41^{klm}	55^{ab}
21	48^{hij}	43^{ijk}	42^{jkl}	33^{n}	42^{d}
$\bar{X}$	57^{a}	56^{ab}	52^{c}	41^{d}	

(b) WD2

Temperature °C	Moisture content %				
	6	8	10	17	$\bar{X}$
-5	58^{ab}	53^{abcde}	46^{efg}	26^{ij}	46^{bc}
4	59^{a}	59^{a}	49^{bcdef}	38^{gh}	51^{a}
10	57^{abc}	59^{a}	56^{abcd}	17^{m}	47^{b}
21	29^{i}	25^{ijk}	24^{ijkl}	8^{n}	22^{d}
$\bar{X}$	51^{a}	49^{ab}	44^{c}	22^{d}	

(c) WD3

Temperature °C	Moisture content %				
	6	8	10	17	$\bar{X}$
-5	57^{b}	42^{bc}	27^{efgh}	17^{ij}	36^{b}
4	53^{a}	41^{bcd}	41^{bcd}	29^{ef}	41^{a}
10	41^{bcd}	44^{bcd}	30^{e}	11^{k}	32^{bc}
21	28^{efg}	18^{i}	10^{kl}	1^{m}	14^{d}
$\bar{X}$	45^{a}	36^{b}	27^{c}	15^{d}	

(d) WD4

Temperature °C	Moisture content %				
	6	8	10	17	$\bar{X}$
-5	2^{hij}	10^{cde}	7^{cdef}	1^{hijk}	5^{bcd}
4	25^{a}	3^{hi}	3^{hi}	4^{fgh}	9^{ab}
10	21^{ab}	7^{cdefg}	10^{cd}	0^{ijkl}	10^{a}
21	7^{cdef}	13^{bc}	3^{hij}	0^{ijkl}	6^{bc}
$\bar{X}$	14^{a}	8^{b}	6^{bc}	1^{d}	

Table 5.1 Mean viability (germination %) for A3 seed; ranked on the basis of LSD as indicated by superscripts.

Analysis of Variance for WD1

Source	DF	SS	MS	F	P
MC	3	1.51019	0.50340	38.98	0.000
Temp	3	1.34234	0.44745	34.65	0.000
MC*Temp	9	0.13003	0.01445	1.12	0.368
Error	48	0.61992	0.01292		
Total	63	3.60249			

Analysis of Variance for WD2

Source	DF	SS	MS	F	P
MC	3	6.62199	2.20733	100.83	0.000
Temp	3	6.73048	2.24349	102.48	0.000
MC*Temp	9	0.80241	0.08916	4.07	0.001
Error	48	1.05078	0.02189		
Total	63	15.20566			

Analysis of Variance for WD3

Source	DF	SS	MS	F	P
MC	3	7.9563	2.6521	141.99	0.000
Temp	3	7.4765	2.4922	133.42	0.000
MC*Temp	9	1.1330	0.1259	6.74	0.000
Error	48	0.8966	0.0187		
Total	63	17.4623			

Analysis of Variance for WD4

Source	DF	SS	MS	F	P
MC	3	5.28008	1.76003	27.55	0.000
Temp	3	0.52343	0.17448	2.73	0.054
MC*Temp	9	5.30296	0.58922	9.22	0.000
Error	48	3.06665	0.06389		
Total	63	14.17312			

Table 5.2 Analyses of variance for A3 seed percentage viability transformed to probit scale.

and 8% with temperatures of -5, 4 and 10 °C were the best and not significantly different from each other; combinations of 10% moisture content with temperatures of -5, 4 and 10 °C were intermediate and combinations of moisture content 17% with all temperatures or of temperature 21 °C with all the moisture contents were the poorest (Table 5.1b).

5.3.3 Withdrawal 3

The effect of moisture content on viability was highly significant ($F = 141.99$, $P < 0.001$). The viability again decreased as moisture content increased and there were significant differences between all four means.

The effect of temperature on viability remained significant ($F = 133.42$, $P < 0.001$) with a decrease from the previous withdrawals but the same trend of mean values as in previous withdrawals.

The effect of interaction between moisture content and temperature on viability was that there was a general decrease of viability with increasing moisture content for all the temperatures. The combination of moisture content 6% with temperature 4 °C was significantly higher than all others. The combination of 6 and 8% with -5, 4 and 10 °C maintained viability better than others, as in previous withdrawals, and the combination of 10% with 4 °C was in the same significance level. The interaction of 17% with all the temperatures and of 21 °C with all the moisture contents again showed very low viability, a combination of 17% and 21 °C being the lowest.

5.3.4 Withdrawal 4

There was a marked reduction in viability compared with the previous withdrawals. Analysis of variance showed the moisture content effect ($F = 27.55$, $P < 0.001$) to be more significant than the temperature effect ($F = 2.73$, $P = 0.054$). Viability at 6% was significantly higher than at other moisture contents; at 8 and 10% viability was intermediate and not significantly different, and at 17% it was significantly lowest (only 1% germination).

The moisture content and temperature interaction effect on viability was statistically significant ($F = 9.22$, $P < 0.001$). The combinations of the moisture

content 6% with temperatures of 4 and 10 °C were ranked highest, and there was no significant difference between them. A moisture content of 6% combined with temperatures of -5 and 21 °C resulted in poor viability but 8% moisture content combined with the same temperatures gave a relatively better result, not significantly different from 10% moisture content at -5 and 10 °C. The only seeds to germinate at 17% moisture content were at -5 and 4 °C (1 and 4% respectively).

5.3.5 Slope of declining viability

Seed death is normally distributed in a given environment. This distribution may be displaced to the left or to the right depending on the condition at which the seed has been stored. The transformation of percentage viability to probit values gives a linear relationship from which viability can be predicted in a specified time. Slopes with initial values were calculated from the probit values by using the standard formula and displayed in Table 5.3. The mean slopes were analysed by analysis of variance using LSD, calculated from Error Mean Square using the formula $2\sqrt{(2\ EMS)/N}$ to compare the difference between treatment means, and ranked on this basis (Appendix 5.2).

The rate of declining viability increased with an increase in moisture content (Table 5.3). In the case of temperature, rate of declining viability was least at storage temperatures of 4 and 10 °C, greatest at 21 °C and intermediate at -5 °C.

Considering the interaction of moisture content and temperature, seeds dried to a moisture content of 6% and then stored at temperatures of 4 and 10 °C were found to have the best longevity. Seeds with 17% moisture content showed relatively rapid decline in viability at -5, 10 and 21 °C.

Analysis of variance was carried out for the mean slopes (Table 5.4). The F value for moisture content effect (45.27) was higher than those for temperature (F = 7.49) and the interaction of moisture content*temperature (F = 9.93) but all effects were highly significant. It appears from the F-values that the moisture content effect was most important.

	Moisture content %				
Temperature °C	6	8	10	17	$\bar{X}$
-5	-5.26^{ijkl}	-4.01^{cd}	-4.38^{cdefgh}	-5.87^{klm}	-4.88^{bc}
4	-2.50^{a}	$-5.09^{efghijk}$	-5.05^{efghij}	-4.95^{efghi}	-4.40^{a}
10	-3.02^{ab}	-4.28^{cdefg}	-4.28^{cdef}	-6.38^{mno}	-4.50^{ab}
21	-4.27^{cde}	-3.91^{c}	-5.90^{klmn}	-6.99^{op}	-5.27^{cd}
$\bar{X}$	-3.76^{a}	-4.33^{b}	-4.90^{c}	-6.05^{d}	

Table 5.3 An analysis of the rate of loss of viability over time. The values refer to the slope of the relationship between germination rate and time, where germination is expressed in probit units: superscripts denote ranking on the basis of LSD, i.e. means with the same superscript are not significantly different.

5.3.6 Comparison of trends of declining viability at different moisture contents

Graphs of declining viability were drawn to compare moisture contents and to see the effect of temperature at each moisture content (Figure 5.1).

A combination of every moisture content with a temperature of 21 °C resulted in consistently declining viability from the beginning of the storage period. The moisture contents of 6 and 8% maintained viability well up to 270 days after which the viability dropped rapidly. With the control moisture content (11%) viability decreased rather steadily, apart from the storage temperature of 4 °C which showed a rapid decrease after 270 days and similarly 10 °C after 180 days. At a moisture content of 17% viability declined relatively quickly followed by a rapid decrease after 270 days. It can be seen that viability dropped significantly at about 300 days after collection.

Analysis of Variance for b(wd0-4)

Source	DF	SS	MS	F	P
MC	3	45.7634	15.2545	45.27	0.000
Temp	3	7.5758	2.5253	7.49	0.000
MC*Temp	9	30.1146	3.3461	9.93	0.000
Error	48	16.1735	0.3369		
Total	63	99.6272			

MEANS

MC	N	b(wd0-4)
6	16	-3.7617
8	16	-4.3272
10	16	-4.9024
17	16	-6.0469

Temp	N	b(wd0-4)
-5	16	-4.8764
4	16	-4.3982
10	16	-4.4949
21	16	-5.2687

MC	Temp	N	b(wd0-4)
6	-5	4	-5.2585
6	4	4	-2.5011
6	10	4	-3.0210
6	21	4	-4.2663
8	-5	4	-4.0050
8	4	4	-5.0911
8	10	4	-4.2993
8	21	4	-3.9135
10	-5	4	-4.3761
10	4	4	-5.0513
10	10	4	-4.2792
10	21	4	-5.9032
17	-5	4	-5.8660
17	4	4	-4.9494
17	10	4	-6.3803
17	21	4	-6.9919

Table 5.4 Analysis of variance for the mean slopes of declining viability lines and mean values of the slope for moisture content , temperature and moisture content*temperature interactions, ranked on the basis of LSD.

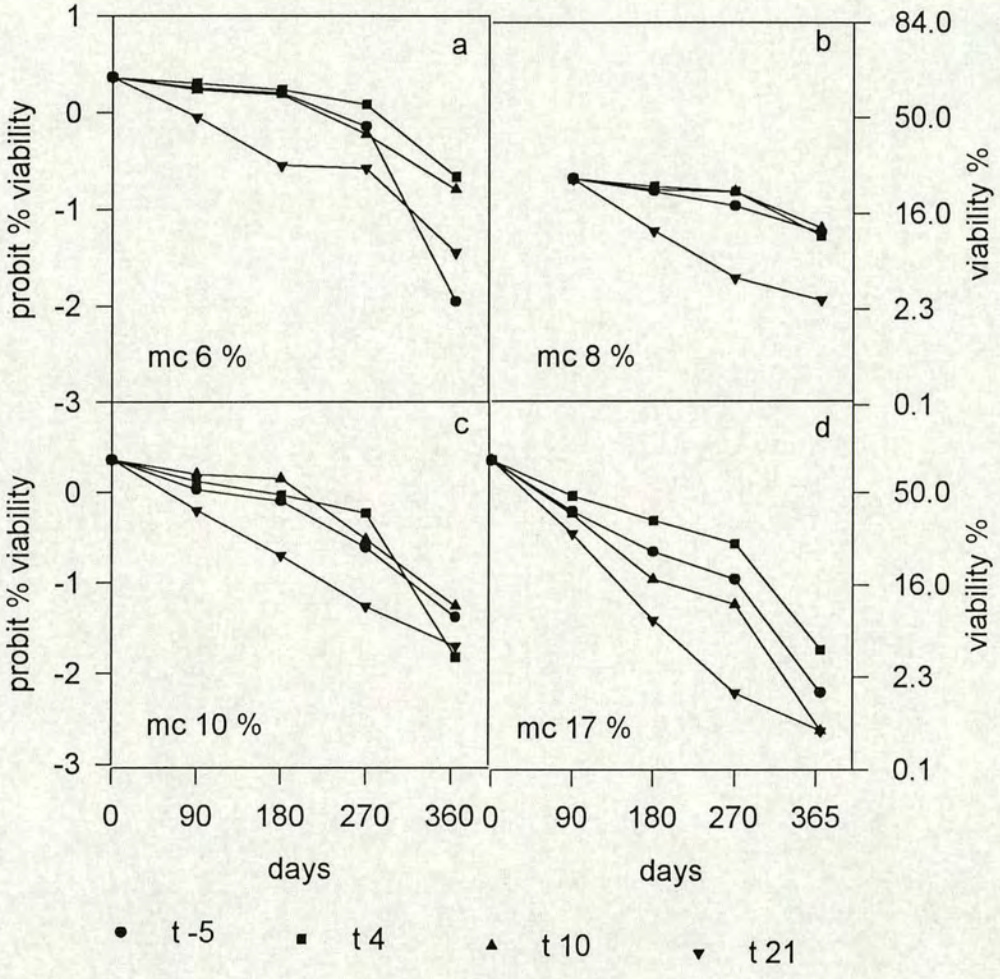


Figure 5.1 Trends of declining viability with time at different moisture contents (mc): separate graphs for storage temperature are shown for each moisture content.

5.3.7 Comparison of trends of declining viability at different temperatures

Graphs of declining viability were drawn to compare storage temperatures, and to see the effect of moisture content at each temperature (Figure 5.2). A combination of every temperature with a moisture content of 17% showed the most rapid decline in viability, generally becoming more rapid after 270 days. At temperatures of -5, 4 and 10 °C seed viability decreased slowly initially and then more rapidly (after 180-270 days) apart from 17% moisture content where viability declined more or less consistently throughout. A temperature of 21 °C also resulted in consistently declining viability from the start of the storage period, most rapidly at 17% moisture content. At -5 °C, after 270 days, seeds with moisture contents of 6 and 17% showed more rapid decrease in viability than seeds with moisture contents of 8 and 10%. At 4 and 10 °C, seed with 6% moisture content showed highest viability after storage for one year.

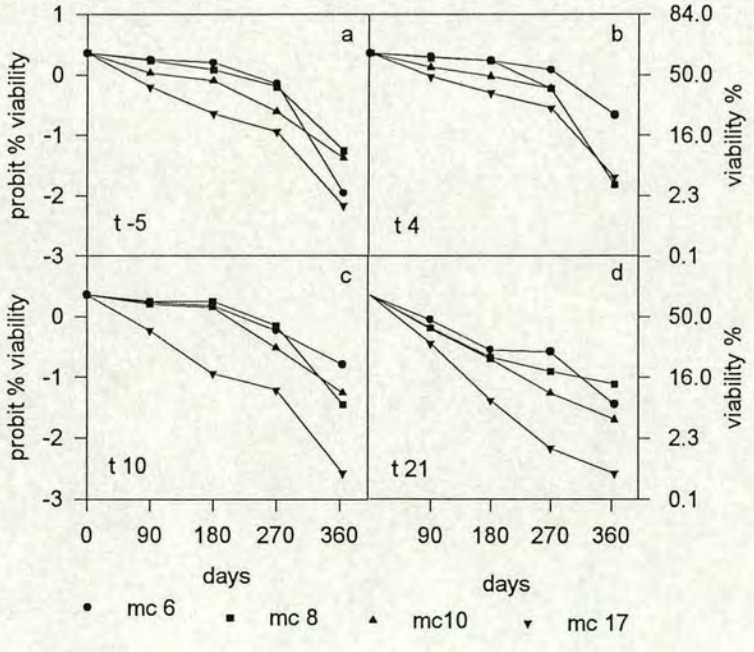


Figure 5.2 Trends of declining viability with time at different storage temperatures: separate graphs for moisture content are shown for each temperature.

5.4 Discussion of storage behaviour (A3 seed)

5.4.1 Moisture content effects

It appears that the seed tolerated desiccation down to 6% moisture content since longevity was relatively better at low moisture content treatments. This is similar to the behaviour of orthodox seeds. Some researchers explain desiccation tolerance in terms of cell membrane stability. Stability of membranes in seeds depends on the ratio of oligosaccharide to sucrose. Oligosaccharide is accumulated by slow drying during seed maturation (Blackman *et al.* (1992) in Lin and Huang (1994)) and the proportions of sugars are controlled by the genetic make up of the species. Recalcitrant seeds are in the process of continuous germination after seeding and hence have a low concentration of oligosaccharides because of ongoing carbohydrate metabolism (Lin and Huang, 1994). In the same experiment Lin and Huang reported that the mole ratio of stachyose + raffinose (oligosaccharides) to sucrose is required to be at least 0.05 to develop desiccation tolerance as seen in orthodox seeds. The same research group found that for *Podocarpus nagi* and *P. macrophyllus* (both recalcitrant species) the above mole ratio was 0.039 and 0.044 respectively. According to Koster and Leopold (1988), sucrose may interact with membrane surfaces as water is removed during dehydration in the presence of oligosaccharide and eventually the membrane is stabilised and protected. They explained this by suggesting that the hydroxyl group of sucrose may replace that of water by hydrogen-bonding to the phospholipid head groups of the membrane and an amorphous 'glass' can be formed which appears to strengthen the cell membrane during drying. By contrast, in a recalcitrant *Guilfoylia monostylis* seed below certain moisture contents (about 43-44%) there is insufficient oligosaccharide present for membrane stabilisation and the deleterious effects of slow drying become apparent (Nkang, 1988). The fact that *Afrocarpus gracilior* tolerated desiccation down to 6% moisture content could indicate that the mole ratio of oligosaccharide to sucrose is within the range of orthodox seed storage behaviour.

When the control seed of *A. gracilior* was rehydrated from 10 to 17% moisture content, particularly at the higher temperature of 21 °C, it lost viability rapidly

compared to other moisture contents (see Tables 5.1 and 5.3). One possible reason for this is that the seed enters the early stages of germination, which cannot be completed for lack of sufficient moisture, i.e. it has become desiccation intolerant. This behaviour again indicates orthodox character. The fact that seeds survived desiccation below 20% moisture content indicates that it is not recalcitrant.

5.4.2 Temperature effects

It is clear that this seed does not tolerate storage at low temperatures, e.g. -5 °C. This indicates non-orthodox character in contrast to the previous section. At temperatures of 10 °C and below the trend of decreasing viability with time was convex, showing rapid fall in viability after 180-270 days storage. This can be explained in terms of cumulative damage: Vossen (1979) reported a similar result with coffee seed (intermediate category) in that seed chilled below 10 °C suffered a rapid loss of viability irrespective of moisture content. By contrast, seeds stored at higher temperature (21 °C) showed a concave trend of decreasing viability with a reduced rate of decline after 180 days storage. This might be explained by the operation of a repair system; it is possible that such a system would operate best at a storage temperature of between 10 and 21 °C and moisture content of between 8 and 17%.

5.4.3 Effect of moisture content and temperature interaction

Most of the treatment combinations showed a constant decrease in viability up to a storage period of 270 days after which there was a rapid decline in viability. The viability of seed dried to 6% moisture content declined considerably at -5 °C after 270 days but less quickly at 4 and 10 °C. This shows that the seeds which have been dried most (6%) were susceptible to damage at lower temperatures, particularly below zero. Ellis *et al.* (1990a) researching on *Coffea arabica*, a species with intermediate seed storage behaviour, found the behaviour to be inconsistent: some individuals of certain seed lots survived considerable desiccation and, at least, short-term storage at subzero temperature whereas seeds of other lots were much more sensitive to damage resulting from desiccation and storage at subzero temperature. In this study viability

of seed dried to a moisture content of 6% at 21 °C kept relatively well but fell drastically after 270 days possibly because the seed had a low metabolic rate with little operation of repair system.

Cumulative damage to cellular organelles may result in seed deterioration gradually or dramatically (Roberts, 1973). He pointed out that during seed deterioration (membrane damage, impaired biosynthesis, greater susceptibility to environmental stress and inability to germinate) the seed accumulates chromosomal damage and evidence suggests that this is a continuous, slow but cumulative process beginning soon, or possibly immediately after harvest. Similarly, Hendry (1993) reported that seeds are in no significant way exempt from the ravage of free radical or oxidative attack, and decline in viability, whether natural or artificial, has long been associated with damage to nucleic acid and cell membranes. Actively respiring seeds seem likely not to suffer from oxidative damage. Seeds hydrated to full imbibition (Basu and Pal, 1980) and moist, ungerminated recalcitrant seeds of *Quercus robur* Hendry (1993) have no such problems as they produce protective enzymes with quenching properties.

However, seeds stored with moisture contents of 8, 10 and 17% at 21 °C showed a constant decline in viability (8% moisture content was the best): this could be due to an effective repair system occurring under these conditions. This is in conformity to the report by Tilden and West (1985) that the mechanism of reversal is probably metabolic because it depends on temperature, moisture content and treatment duration. Repair may occur at moisture content values below full hydration, as low as 20-25% in onion and 15% in lettuce.

5.4.4 Conclusions

The main points raised in the above discussion are as follows.

1. The seed of *A. gracilior* demonstrates intermediate behaviour. Although it withstood desiccation to 6% indicating orthodox character, its viability fell rapidly after 270 days storage at this moisture content showing that it is not truly orthodox. Equally, it is not recalcitrant because the majority of seeds

survived desiccation below 20%. The fact that seed was damaged at low temperatures, particularly subzero, again indicates that it is not orthodox.

2. The storage behaviour of *A. gracilior* is similar to that of some tropical species of intermediate category, such as *Coffea arabica* and *Carica papaya*. It shares the same ecological conditions as coffee.
3. Seed dried to a lower moisture content (6%) and stored at lower temperatures (e.g. -5°) showed a drastic decline in viability on a subsequent storage. The optimum storage condition appears to be between the moisture content of 8 and 17%, close to 8 and between the storage temperature of 10 to 21 °C close to 10 °C.

5.5 Comparison of 3 storage experiments: A2 seed tested by tetrazolium and germination, and A3 seed tested by germination

The effects of moisture content and temperature on seed viability are shown in Figures 5.3 and 5.4 respectively, comparing tetrazolium tests on A2 seed with germination tests on A2 and A3 seed. The discussion compares tetrazolium and germination tests on the one hand, and the two seedlots on the other.

5.5.1 Comparison between tetrazolium and germination methods

Tetrazolium values are higher than germination values when measuring viability. As TZ and A2 experiments share identical seed they were expected to show small differences. Several researchers (Gaspar and Nagy, 1981; Moore, 1985; Leadem, 1995) indicated that tetrazolium results are higher than the corresponding germination values because TZ is independent of dormancy. In a highly dormant seed TZ could give a poor prediction of germinability.

In these experiments, the trend of viability is found to be similar. Comparison between the three experiments (TZ, A2 and A3) shows that viability decreases with an increase in moisture content (Figure 5.3) and shows a peak in the region of temperature between 4 and 10 °C (Figure 5.4).

Analysis of variance between the slopes of declining seed viability over time in relation to storage moisture content and temperature indicated that there are no significant differences between the three experiments ($F = 1.40$, $P > 0.27$ for Experiment*MC; $F = 1.81$, $P > 0.15$ for Experiment*Temperature: Table 5.5). This means that the tetrazolium and germination methods of determining viability are reasonably correlated within the experimental range of storage conditions: the increased germination values at 8% and 21 °C may be due to the operation of repair systems, as discussed earlier.

In addition to dormancy, incubation temperatures may cause a wide difference between results of tetrazolium and germination tests. For example, *A. gracilior* can stain with tetrazolium over a wide range of temperature (say between 20 and 35 °C), but is sensitive to temperature for germination, the best being 30 °C.

Lack of skill in interpreting the results of tetrazolium tests is another source of difference between the two methods: the pattern and intensity of staining varies and long experience is necessary for consistent results.

Source	**DF**	**SS**	**MS**	**F**	**P**
Expt	2	45.5411	22.7705	39.87	< 0.001
MC	3	67.5127	22.5042	39.40	< 0.001
Temp	3	24.7807	8.2602	14.46	< 0.001
MC*Temp	9	19.6096	2.1788	3.81	0.008
Expt*MC	6	4.8129	0.8022	1.40	0.266
Expt*Temp	6	6.2051	1.0342	1.81	0.154
Error	18	10.2807	0.5712		
Total	47	178.7428			

Table 5.5 Analysis of variance for the slope of declining viability with time for the three seed storage experiments combined.

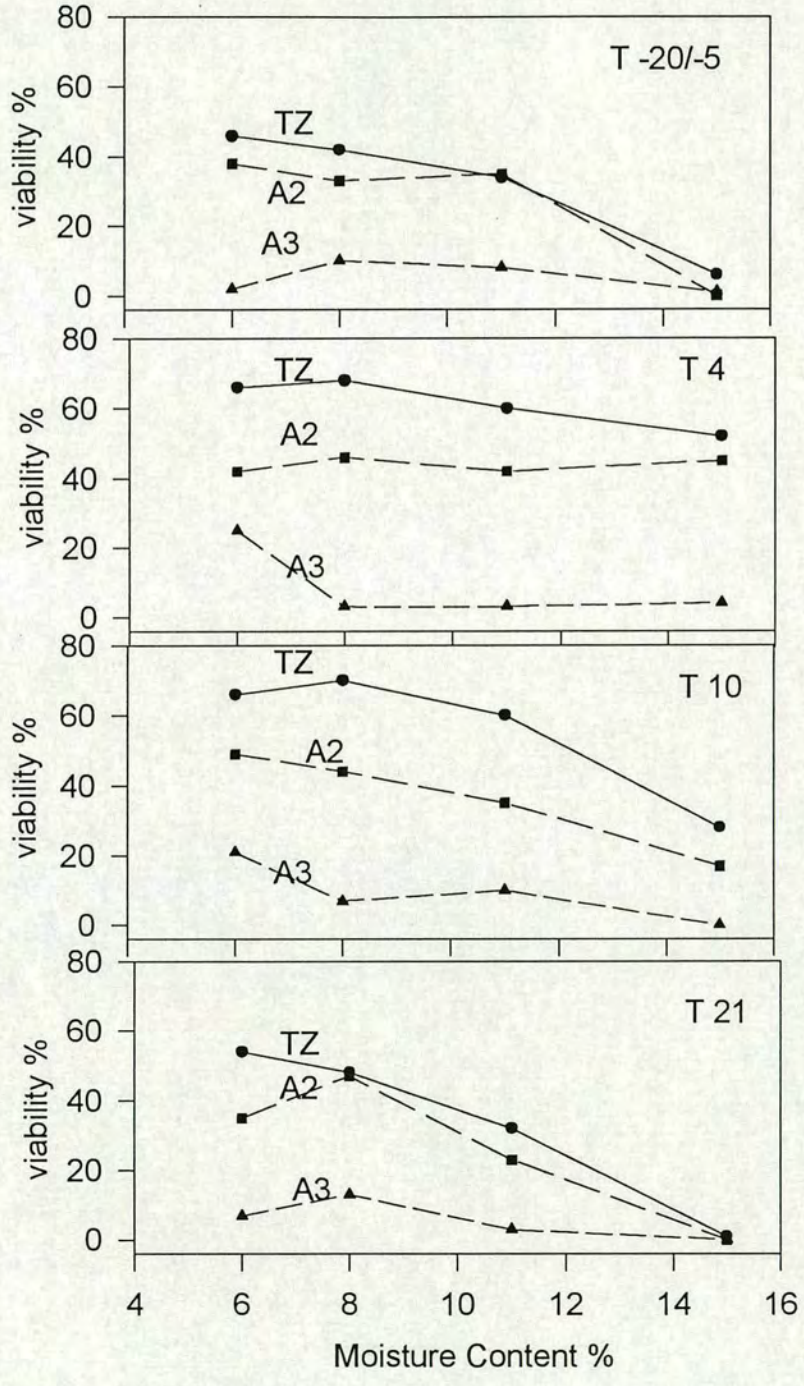


Figure 5.3 The effect of moisture content on viability of *A. gracilior* seed after 12 months in storage. Viability was measured by TZ (for A2) and by germination (for A2 and A3). T -20/-5 °C means that A2 seed was stored at -20 °C, while A3 was stored at -5 °C.

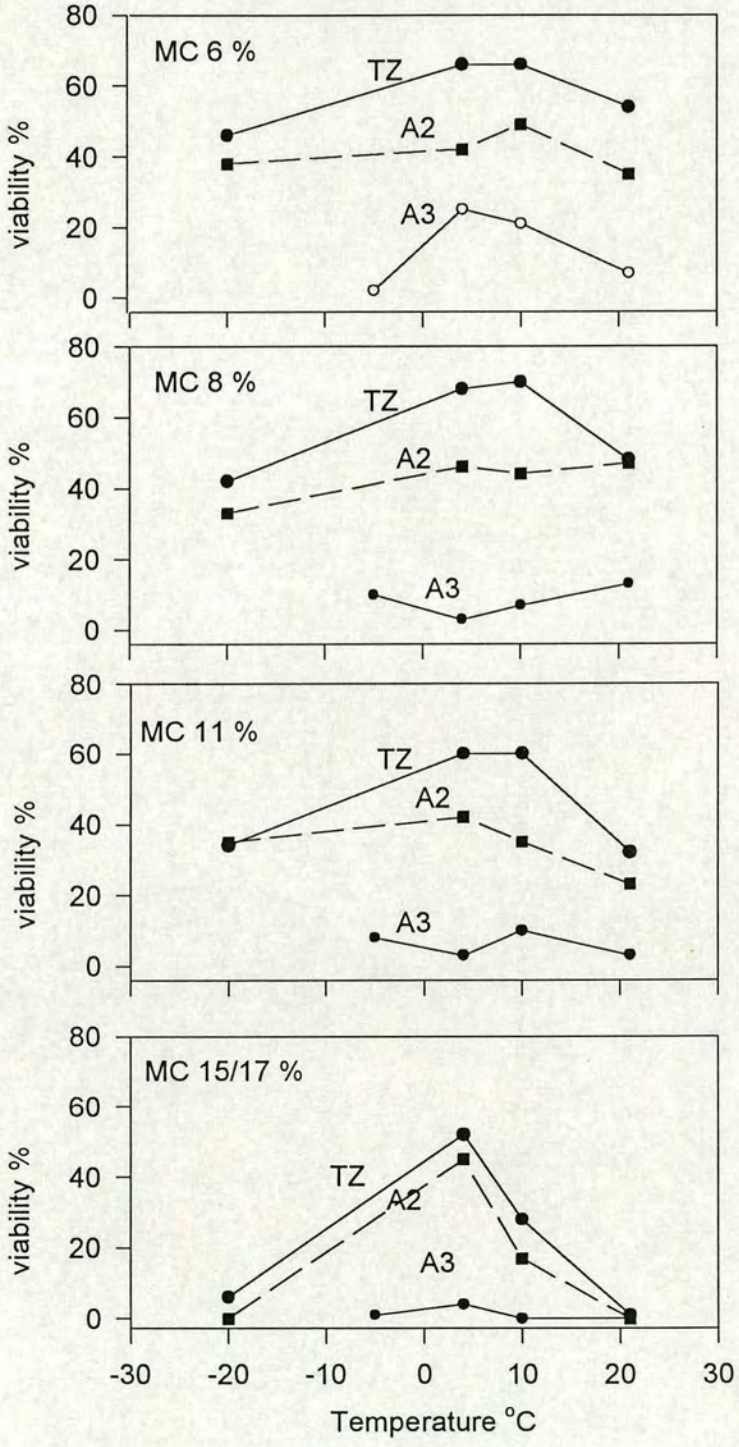


Figure 5.4 The effect of temperature on *A. gracilior* seed after 12 months in storage. Viability was measured by TZ test (A2) and germination (A2 and A3). A2 seed was rehydrated to 15% moisture content while A3 seed was rehydrated to 17%.

5.5.2 Comparison of A2 and A3 seed lots

Examination of Figures 5.3 and 5.4 suggests that the main difference between the three experiments came from the seed lots. The two seed lots had different viability (germination method) on receipt (81% for A2 and 64% for A3). However, A2 still showed a viability of 49% (with its best storage combination) two years after collection while A3 only showed a viability of 25% after one year. The possible sources of the differences between the two seed batches are now considered.

(a) Genetic variation

Conifers in general show more variations within the species than do the broad-leaved plants (Dr C N Page, pers. comm., 1995). It appears that *Afrocarpus* has a wide genetic base and might interbreed with other East African *Podocarpaceae*. *Afrocarpus* are intermediate in taxonomic position between south-east Asian genera *Nageia* and *Retrophyllum* on one side and African *Podocarpus* on the other (Page, 1988). It could therefore be that the genetic variability is high for significant characteristics within the same provenance. Ellis *et al.* (1991a) worked on different cultivars of *Coffea arabica* and pointed out that desiccation sensitivity varied among lots within cultivars. Similarly, Hong and Ellis (1992) indicated that differences in response to given storage environments could have arisen as a result of the effect of either genotype or provenance and hence a seed lot should encompass a wide range of genotypes.

(b) Seed quality

The environmental conditions at the time of seed formation can have a great influence on the quality of a seed. The right temperature, adequate soil moisture for plant growth, enough light and adequate length for the growing season can determine the quality of a seed (Quinlivan *et al.*, 1987). Regarding A2 and A3 seed lots, one of the possible causes for the differences in their viability could be environmental stress.

Seed immaturity is one of the possible causes of low quality seed. If the seeds are harvested before they have accumulated storage reserves, the cell membranes cannot withstand desiccation. Experiments on immature (green) and mature (yellow) seeds of *Coffea arabica* indicated that green berries were most sensitive to the initial desiccation treatment, more than half the population being killed when dried to 11.6% moisture content, while yellow berries showed the greater tolerance to desiccation (Ellis *et al.*, 1991a).

Seed extraction and drying are very important steps to procure a quality seed. Pre-soaking prior to seed extraction is practised in Ethiopia in order to soften the pulp for easy removal by hand. Regarding *Afrocarpus* seeds, whether or not pre-soaking has any beneficial effect is not yet clear. Rehydrated seed from 11 to 15% moisture content in a saturated atmosphere and stored at 4 °C had more or less the same viability after one year of storage compared with the seed dried down to 8% moisture content and stored at 4 or 10 °C. Researchers have different opinions about pre-soaking on some other species. Chowdhury and Chouduri (1987) reported that pre-soaking followed by dehydration caused an improvement in membrane integrity in jute. On the other hand, Bewley (1979) pointed out that pre-soaking followed by dehydration can gradually shift the seed from desiccation tolerant to desiccation intolerant. Pre-soaking might cause physical injury, in that cellular membranes of the fresh seed offered great resistance to the rapid entry of water into the cell of fully immersed seed and suffered greater damage than the relatively leaky membranes of the older seed (Basu and Pal, 1980).

In *Afrocarpus* seed, there is a gradual loss of moisture before the seeds are shed followed by a rapid loss of moisture after shedding. Fresh seed from which the pulp had been removed and dried in the shade had a moisture content of 12% in three days. This seed was stored in Edinburgh at 4 °C and lost its viability in a period of six months. It has been observed in several supportive experiments of this study that the embryo of such a seed could still contain a high moisture content and it needed more time to equilibrate with the surrounding tissues. Measurements on two *Afrocarpus* seed lots A4 and B2 (Table 5.6) indicated that the seed coat and the gametophyte have different composition: for example, the moisture content of coat and

gametophyte were 14.4 and 6.6% respectively (averaged for both seed lots) before the embryo was equilibrated. After equilibration at 15 °C and 15% rh for 15 days the oil content was found to be 0.5% for the seed coat but 65.5% for the gametophyte, and the moisture content to be 6% and 2.4% respectively. Thus, the moisture content ratio of coat to gametophyte increased from 2.2:1 before equilibration to 2.5:1 after equilibration. This shows that there is a moisture content gradient in the seed; the embryo was wetter than expected at the end of the ordinary drying method. Although the moisture content of the gametophyte is less than the seed coat, it might have insulated the embryo from losing some moisture. The water potential of the embryo could be higher than that of the surrounding gametophyte or seed coat. This indicates that slow drying for about three weeks in a dry condition of about 25% rh and 15 °C is important to bring the embryo to a stable storage condition. This is in conformity with the result obtained by Grout *et al.* (1983) that if one considers the separate constituent tissues rather than the whole seed at low water content it is apparent that the embryo retains significantly more water than the surrounding endosperm. In the same way, some of the damage could be due to moisture not having equilibrated within the seed so that the moisture content of the embryo remained high (Ellis *et al.*, 1990b).

(a) Moisture content before equilibration

Species	**Coat**	**Gametophyte**
A. gracilior (A4)	14.3	6.7
A. gracilior (B2)	14.5	6.4

(b) Equilibrium moisture content (EMC) and oil content

	EMC% at 15 °C and 15% rh		**Oil%**	
Species	**Coat**	**Gametophyte**	**Coat**	**Gametophyte**
A. gracilior (A4)	5.9	2.3	0.3	66
A. gracilior (B2)	6.1	2.5	0.7	65

Table 5.6 Moisture content before, and moisture & oil contents at the equilibrium point in two seed lots of *A. gracilior* (A4 and B2).

Seed size is also important for quality. It is generally true that large seed has accumulated more storage reserves than small seed and therefore has more potential for germination. However, large seed takes relatively more time to dry and precautions should be taken before storing such seed. In this study, it has been noted that the size of A3 was 1.5 times the size of A2. However, in terms of longevity after storage in the same conditions, A2 seed was able to show germination of about 50% two years from the time of collection while A3 (after one year) showed only 25%. Ellis *et al.* (1991c) found a similar result with intermediate storage category seed of oil palm (*Elaeis guineensis*) that the largest seed cultivar was the most desiccation sensitive.

5.6 Conclusions and Recommendations

With regard to this work, it is now possible to predict the optimal combination of storage moisture content and temperature. Further work still remains to be done, particularly concerning pre-storage conditions for example.

1. It is very important that only mature seeds should be collected. Care should be taken not to use seed for long term storage in particular unless it is well known that it was collected mature.
2. Proper handling is necessary, especially during temporary storage in the field and during transporting. Seeds should be contained in small bags to prevent moisture build-up and high temperature which cause seed deterioration in a short period of time.
3. Seed extraction should be based on the objective of collection. If it is for long-term storage for genetic conservation, no pre-soaking is required, but desiccation should continue until the moisture within the seed is equilibrated. If the objective is seedling raising for the current plantation programme, pre-soaking prior to extraction may be considered.
4. The optimal storage moisture content appears to be about 8%.
5. The optimal storage temperature appears to be about 10 °C.

Seed dried to 6% was best stored at around 10 °C: seed dried to 8% stored well at a wide range of temperatures, between 4 and 20 °C. On the temperature side, 4 °C maintained viability well for a wide range of moisture content, 6-15%, although A3 seed appears to be damaged at 6%.

CHAPTER 6

Micropropagation of *A. gracilior*

Introduction

Although the potential benefit of clonal propagation in reforestation has long been recognised, it has not been well standardised on a world-wide basis. Forest resources are being depleted at a faster rate than they are being replaced. Unless all the potential regeneration techniques are applied, the very existence of certain tree species will be in jeopardy.

There is a high potential to achieve objectives in the current demand of forest resources by using clonal propagation. While a rooted cutting can produce a single plant from which, several years later, further cuttings are available, even the most limited *in vitro* culture systems can produce several axillary as well as adventitious shoots (Thorpe *et al.*, 1991). Gupta *et al.* (1993) reported that forest species with high-value genotypes can be gained by rapid multiplication through micropropagation.

The plant multiplication rate can be geometrically increased by removing the apical dominance of the growing tip so that axillary buds can be initiated at leaf axils. For example, stem segments with removed apical half-length needles from mature *Pseudotsuga menziesii*, formed large numbers of axillary buds *in vitro*, many of which rooted (Bonga and Aderkas, 1992). A species of this type can have a high potential for micropropagation as all the developing buds could be subsequently used as the growing shoots. Subsequent subculturing is needed to increase the number of useable shoots. In micropropagation, very small plants can be used, which is one of the advantages over *ex vitro* propagation. It saves time and space.

Propagated shoots can be made to root in a suitable environment, with or without growth regulators. There are many physical and chemical factors that promote rooting *in vitro*. The physical factors: water stress, high temperature, activated charcoal, oxygenation and reduced light intensity stimulate rooting, (Gaspar and Coumans (1987) in Bonga and Aderkas, 1992). A high auxin/cytokinin ratio is necessary for root initiation, and the auxins IBA and NAA are often more effective than IAA because of the instability of the latter (Bonga and Aderkas, 1992). Roots

are induced on Murashige and Skoog (MS) or Woody Plant Medium (WPM) with Naphthalene Acetic Acid (NAA) or Indole Butyric Acid (IBA) at various concentrations depending on the type of species. According to Bose and Mandal (1973), treatment of cuttings, especially in the tree group including *Podocarpus macrophyllus*, with IBA or NAA improved the rooting percentage. *In vitro* cultures were initiated from seedlings of *Faidherbia albida*, on MS medium supplemented with various combinations of BA at 10^{-7} M and NAA at 10^{-7} or 10^{-8} M, and rooting and vigorous growth were most successful on medium supplemented with 10^{-7} M NAA alone, on which 87% of the shoots formed roots (Ruredzo and Hanson, 1993). Wainwright and Tutt (1987) reported better callus formation in *Betula pendula* shoots following a three second dip in high concentrations of IBA.

Cheng *et al.* (1992) reported that variation in the level of sucrose had significant effects on the rooting of *Eucalyptus sideroxylon in vitro*. Rooting was good at concentrations of 5% but inhibited at 9%. Activated charcoal was found to be effective in the rooting of axillary shoots of *Quercus crispula in vitro* after basal treatment with IBA for three seconds (Sengi, 1990).

Incubation conditions of cultures are important in growth and development of the roots. According to Pevalek-Kozlina and Kelaska (1989) *in vitro* rooting of *Prunus avium* gave the best result of 70-90% when placing the shoot on agar solidified modified WPM supplemented with 2% sucrose and 4.9 mM IBA under a regime of 16/8 h light/dark photoperiod at 24 °C and 20 °C and illuminated by white fluorescent lamps.

On the other hand, it has been reported that the concentration of basal media is important. Jackfruit (*Artocarpus heterophyllus*) rooted at 80% on half-strength MS salts supplemented with 5.37 mM NAA and 4.92 mM IBA (Roy *et al.*, 1993).

Aqueous extracts of some plants can inhibit germination and growth of adjacent seeds. According to Chou and Waller (1980), aqueous extracts of leaves, stems and roots of *Coffea arabica* significantly inhibited the seed germination of and radicle growth of rye grass, lettuce and some other species. Similarly, in Chapter 2 of this work, it has been reported that aqueous solution from the seed coat of *Afrocarpus gracilior* could completely inhibit germination of wheat seed. However, in this

chapter, an aqueous extract of the seed has been applied *in vitro* to growing shoots of its own species for an observation on effect on growth and rooting.

Plant material can not be transferred directly to *in vitro* conditions as there are generally contaminating fungi and bacteria present in the substance. These have to be removed by surface sterilisation. There are a wide range of explants possible that might act as the starting point for a micropropagation process. That being investigated in this experiment is the seed and the germinating seed. Several methods were used to obtain explants for subsequent experiments.

6.1 Experiment 1: Culture initiation, maintenance and multiplication

6.1.1 Materials and Methods

In this experiment, 49 clones of *A. gracilior* were established either from seed or germinating seeds using various sterilisation methods.

Four clones were obtained from 50 seeds of A1 seed lot, collected in Lagalencha, Adaba, Ethiopia, in January 1993. The explants were surface sterilised (Appendix 6.1). Fifty explants were transferred to tubes of MS (1962) basal medium (Appendix 6.2) using standard sterile techniques. Some of the explants were gently pushed into the medium until half buried with the micropylar end facing the air space. Others were cultured with the micropyle end buried in the agar. The established cultures were divided into two groups of 25 cultures and incubated in the light (81 μmol photons m^{-2} s^{-1}) or the dark, both at 20 °C.

Thirteen clones were obtained from 25 seeds of A2 seed lot, collected from the same place and at the same time as A1 and two clones were obtained from 25 seeds of B1 seed lot, collected in Sole-Shashemene, Ethiopia, in November 1992. The explants were surface sterilised (Appendix 6.1). The explants were transferred to 10 × 25 cm, flat bottomed soda glass tubes containing 15 mls of moist sterile sand. Each of the explants was half buried in the sand medium, using standard sterile techniques in the laminar flow cabinet. The tubes were capped with aluminium caps and sealed with cling film. The cultures were incubated at 28 °C in the dark and germination was assessed at intervals for six weeks.

The shoots from the explants that germinated were excised and transferred to MS basal medium.

Five clones were obtained from 135 *ex vitro* germinated young shoots of A2 seed lot. The explants were sterilised with calcium hypochlorite (Appendix 6.3).

Twenty-five clones were obtained from 141 shoots germinated *ex vitro* of A2 seed lot by surface sterilisation of young shoots with mercuric chloride (Appendix 6.4).

The sterilised explants were inoculated into MS basal medium in the laminar flow cabinet using standard sterile techniques. The root end of the hypocotyl was discarded and each shoot was pushed into the medium leaving the growing point and the leaves in the air above the medium in the tube. The tubes were capped with aluminium caps and sealed with cling film. The cultures were incubated at 20 °C in the light.

The growth and development of 17 of the established clones, obtained by germination of surface sterilised seeds, were observed over six subcultures of six weeks each. The established shoots were subdivided at each subculture into apical and stem segments and transferred to fresh MS basal medium.

6.1.2 Results

The cultures did not produce axillary buds during normal growth and development. However, on subculture, the apical segment continued growth as usual without producing any axillary buds whereas the stem segments, initially with no buds, were observed to initiate axillary buds in the axils of leaves at the top of the stem segment. The number of buds initiated could vary from 0 to 3, with 1 most frequent (Figure 6.1).

	Subculture Number					
Clone	**1**	**2**	**3**	**4**	**5**	**6**
1	1	3	8	18	16	26
2	1	1	1	1	1	3
5	1	3	5	7	4	6
6	1	2	6	12	19	42
7	1	3	5	6	6	10
8	1	3	5	5	1	2
9	1	1	1	2	3	4
10	1	4	6	8	16	20
11	1	4	12	20	20	38
12	1	3	6	6	2	4
13	1	4	5	8	8	16
14	1	4	11	18	13	28
15	1	3	6	11	11	20
16	1	3	5	8	13	27
17	1	4	8	13	14	32
18	1	3	4	3	1	1
19	1	3	4	6	4	6

Table 6.1 The multiplication of ramets of 17 selected clones of *A. gracilior* over six culture periods of six weeks each. Clones 3 and 4 died during the culture period.

The data for the multiplication of the selected clones is shown in Table 6.1. Some clones had good growth and multiplication, e.g. clones 6 and 11, while some were found to have low multiplication rate, e.g. clones 2 and 8. Some clones, e.g. 5, 8 and 14, decreased in ramet numbers between subculture numbers 4 and 5. This happened due to death of shoot cultures, non-production of axillary buds and rejection due to contamination.

Data for subculture 6 were analysed for any significant difference between clones. The Chi square test indicated that there was a significant difference between clones, $\chi^2 = 174$, compared with the critical value of $\chi^2_{16}(0.05) = 26.3$.

Spontaneous rooting was observed in the various subcultures. The level of rooting varied widely, e.g. at the end of the fifth subculture 74% of clone 6 was rooted whereas only 25% of clone 10 was rooted.

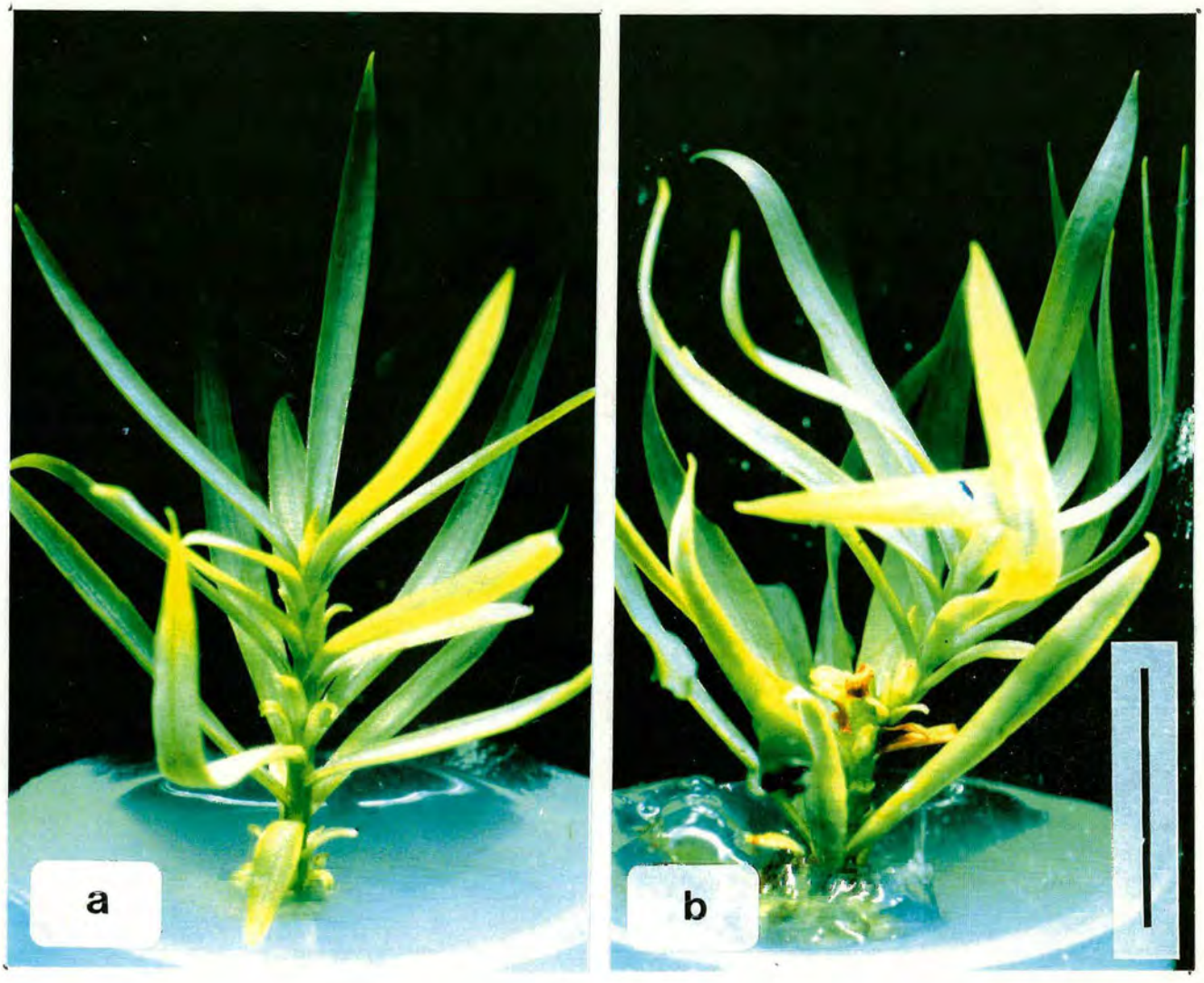


Figure 6.1 Established shoot cultures of *A. gracilior* (a) shoot tip, (b) decapitated shoot (nodal segment) with axillary shoot (bar: 1 cm).

6.1.3 Discussion

This is a simple multiplication technique that works for the majority of clones of this species. The low multiplication rate might be due to a combination of low growth and/or low or no bud initiation. In normal cultures, i.e. those with an apical bud present, there was no axillary bud initiation on the extending segments. Under field conditions in the shade, internodal height could be about 25 cm, whereas it is about 10 cm in the direct sunlight. The pattern of stem growth in the field condition reveals that there is an apical extension followed by a cessation of growth for some time

followed by the formation of whorls of buds from which the apex re-extends. It is possible that the shoot cultures did not extend sufficiently for this bud initiation process to occur.

It was interesting to note that buds were initiated on pieces of stem where the influence of the apical bud had been removed. This bud initiation was not induced by applied plant growth regulators as the medium was hormone free. The anatomy of this bud initiation was not investigated but it is possible to speculate on the mechanism. It is possible that there are cells in the axils of the leaves that are capable of forming a bud but their development is suppressed by the dominance of the apex. It is when this dominance is removed that development can occur. It is also likely that when the axillary buds have formed, they in turn exert dominance over the rest of the stem and prevent axillary bud development below. This is in agreement with the observation that axillary buds were initiated in the topmost region of the stem segment and not along the whole length.

It was found that in this experiment, subdivision and subculture of shoot cultures resulted in a culture with a dominant apex and a culture with no apical bud that subsequently developed an axillary bud or buds. This is, therefore, a method of multiplication by which at least two growing points can be obtained from one shoot after each subculture.

This technique of multiplication:

(a) gives an advantage of using small pieces of tissues compared to macropropagation;

(b) might allow us to reach large numbers of plants in a relatively short period of time.

The technique that has been developed for *A. gracilior* is very unusual in that axillary bud induction occurs after removal of apical dominance without applied plant growth regulators. It is far more common in a very wide range of species for bud induction to occur only when plant growth regulators are applied.

It has been reported that axillary bud induction using plant growth regulators maintains genetic stability better than bud production by organogensis (McCown and McCown, 1987). It is possible to speculate that the technique developed for

A. gracilior could maintain genetic stability even better as no applied growth regulators are involved.

It was observed that spontaneous rooting occurred and it varied between clones. It is possible that *in vitro* rooting of this species might be increased by plant growth regulator treatment. This will be reported later (Section 6.3).

There are a number of reasons why this simple multiplication technique might be implemented in the developing countries of East and South Africa where *A. gracilior* is an important forest species. At present, factors such as problems with seed supply, seed dormancy and viability can lead to restricted amounts of material being available for reafforestation.

6.1.4 Conclusions

1. The technique is simple and applicable for the majority of the clones.
2. At least one bud is initiated in the stem axil when the influence of apical bud is removed.
3. Growth regulators are not involved for bud initiation, hence, genetic stability can be maintained much better than in species where growth regulators are used.

6.2 Experiment 2: Bud induction and growth with plant growth regulator treatment

6.2.1 Materials and Methods

The source of explants for this experiment was the selected clones, multiplied with the techniques described in Experiment 1 of this chapter.

Eight clones that demonstrated good growth and multiplication were selected from the 17 clones used in Experiment 1.

Shoot cultures were treated with four different media.

The four treatments were:

1. MS basal medium with no hormone (MS);
2. MS basal medium containing 2 mg/ℓ BA (BA);

3. MS basal medium containing 0.1 mg/ℓ NAA (NA);

4. MS basal medium containing 2 mg/ℓ BA + 0.1 mg/ℓ NAA (BN).

The hormone containing media were prepared by dissolving the required quantity of either BA, NAA or BA + NAA in 1 MNaOH and adding to the MS basal medium (Appendix 6.2).

Each ramet was divided into two segments, i.e. a top section bearing the apical bud and a bottom section with no buds and transferred to the treatment media.

The number of replications on the four media varied in different clones, i.e. two for clones 10 and 15, four for clones 1, 11, 14 and 17 and six for clones 6 and 16. Because the amount of replication differed between clones, sums of squares and estimated effects for NA, BA, etc have to be adjusted for clones. Finney (1980) describes this type of adjustment for unbalanced data.

The cultures were completely randomised on culture trays and incubated in the light at 20 °C. The cultures were assessed for bud initiation and height growth. Shoots were transferred to hormone free medium and observations were made.

6.2.2 Results

6.2.2.1 Bud initiation

	Hormone							
Clone	**MS**		**NA**		**BA**		**BN**	
	B	**T**	**B**	**T**	**B**	**T**	**B**	**T**
1	0.00	1.00	0.50	1.00	14.00	8.00	7.50	5.00
6	1.30	1.00	1.60	1.00	7.30	4.60	12.60	3.00
10	1.00	1.00	1.00	1.00	8.00	9.00	5.00	7.00
11	1.50	1.00	1.00	1.00	11.50	4.50	9.50	5.00
14	0.50	1.00	1.00	1.00	9.00	9.50	12.00	4.50
15	0.00	1.00	1.00	1.00	8.00	6.00	8.00	4.00
16	2.00	1.00	2.00	1.00	9.70	7.00	9.70	5.30
17	1.00	1.00	1.00	1.00	10.00	5.50	7.00	3.50
$\bar{X}$	1.06	1.00	1.20	1.00	9.75	6.56	9.13	4.50

Table 6.2 Mean of axillary buds initiated by the hormones on bottom (B) and top (T) segments of each clone after six months.

It can be seen from Table 6.2 that treatment with plant regulators, particularly BA, improved the number of buds initiated in the axils of leaves of both tip and base cuttings. Analysis of variances were calculated for clones, hormones, segment types and their interactions to see the effects of these factors (Table 6.3).

Analysis of variance for number of buds

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	7	19.740	19.740	2.820	0.65	0.711
NA	1	9.031	12.001	12.001	2.77	0.101
BA	1	1352.000	1157.001	1157.001	267.48	0.000
Type	1	144.500	89.590	89.590	20.71	0.000
Clone*NA	7	29.323	29.323	4.189	0.97	0.462
Clone*BA	7	26.021	26.021	3.717	0.86	0.543
Clone*Type	7	31.604	31.604	4.515	1.04	0.410
NA*BA	1	12.500	16.519	16.519	3.82	0.055
NA*Type	1	8.000	4.185	4.185	0.97	0.329
BA*Type	1	124.031	86.519	86.519	20.00	0.000
Clone*NA*BA	7	30.021	30.021	4.289	0.99	0.446
Clone*NA*Type	7	37.271	37.271	5.324	1.23	0.299
Clone*BA*Type	7	26.073	26.073	3.725	0.86	0.542
NA*BA*Type	1	5.281	2.090	2.090	0.48	0.489
Clone*NA*BA*Type	7	31.990	31.990	4.570	1.06	0.402
Error	64	276.833	276.833	4.326		
Total	127	2164.219				

Table 6.3 Analysis of variance for bud inductions by hormones on bottom and top segments compared to the control (MS). Note that BA and NA represent the effects of adding BA or NA respectively to a medium, and hence have each 1 degree of freedom.

There was no significant difference in the number of axillary buds initiated between clones across treatments. The difference between the effect of NAA and BA was highly significant ($P < 0.001$). Bud initiation following treatment with BA was highly significant ($P < 0.001$) whether used alone or in combination with NAA ($P < 0.06$). NAA alone had no significant effect on bud initiation.

BA promoted the initiation of buds in every leaf axil in the bottom segments. In tip segments, buds were not necessarily initiated in every leaf axil. The presence of NAA did not affect the mode of action of BA.

The difference in bud initiation between stem types was highly significant ($P < 0.001$). The average difference between tip and base segments in the MS and NAA alone treatments was not significant (LSD 1.47). However, the difference between tip and base segments when the cultures were treated with BA was highly significant ($P < 0.001$).

The cultures treated with BA were normal initially but, during treatment, the stem tissues became considerably swollen, particularly in the basal region of the stem segment (Figure 6.2). In the latter stages of treatment, bud initiation was not confined to the leaf axils but occurred also directly on the stem tissue. However, the BA appeared to inhibit subsequent growth and development of the initiated buds.

Figure 6.2 Lateral development and bud initiation on the bottom segment in BA containing medium (bar: 1 cm).

6.2.2.2 Height increment

The growth of the cultures in the various treatments was measured for the first six weeks of the experiment (Figure 6.3).

In bottom cultures, no growth was observed in NAA and MS treatments until measurements were made after two weeks. The extension growth was subsequently linear with NAA at a faster rate. No growth was observed in BA and BA/NAA until measurements were made after four weeks. The subsequent growth in both treatments was very poor.

All top cultures grew reasonably well. The growth in the NAA and MS treatments was more or less linear. The apical extension in the BA/NAA mixture was possibly linear but at a slower rate. The extension in the BA treatment was fairly good but subsequently slowed down.

	Hormone							
Clone	**MS**		**NA**		**BA**		**BN**	
	B	**T**	**B**	**T**	**B**	**T**	**B**	**T**
1	0.00	14.00	2.50	16.00	0.00	5.50	0.50	7.50
6	5.67	13.33	12.00	9.00	2.00	2.67	1.33	18.00
10	20.00	2.00	21.00	9.00	0.00	3.00	2.00	4.00
11	1.50	15.00	5.50	10.00	2.00	2.50	0.50	3.50
14	9.00	4.50	14.00	13.00	0.00	2.00	0.00	1.50
15	0.00	4.00	4.00	5.00	0.00	4.00	0.00	2.00
16	9.33	10.00	10.67	12.00	1.00	3.67	0.00	2.67
17	1.00	9.00	8.50	17.00	0.00	2.50	0.50	3.00
$\bar{X}$	5.50	10.06	9.63	11.81	0.81	3.19	0.56	6.19

Table 6.4 Mean height increment (mm) six months after the explants were in culture.

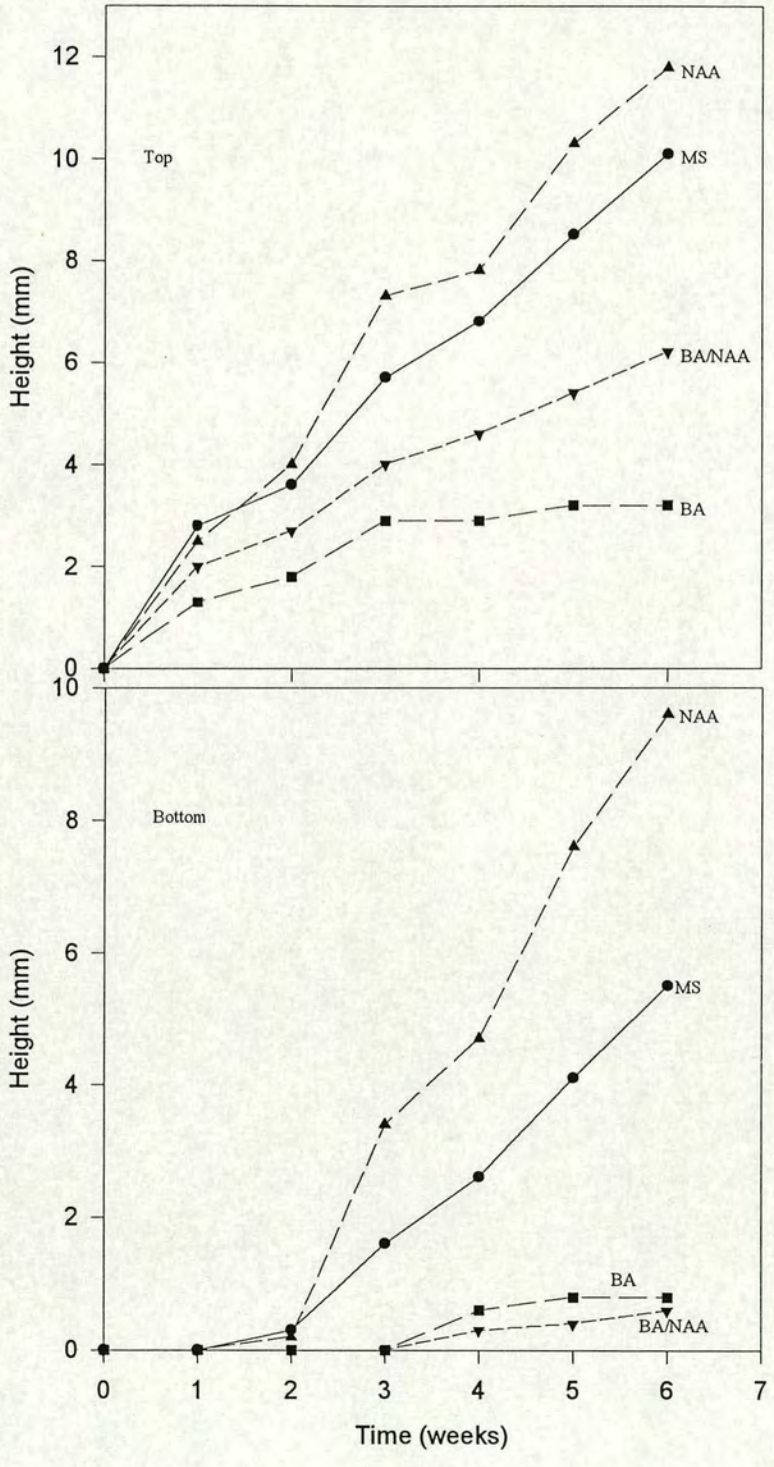


Figure 6.3 Extension of bottom and top cultures on different hormone containing media.

Height increments for top and bottom segments in the various treatments (Table 6.4) were analysed after the explants were in culture for six months (Table 6.5). The increment was the difference between the last assessment and the assessment at zero time.

Statistical analysis indicated that the hormones and the type of segments (bottom/top) had significant effects on height increment. Height differences between clones were not significant in general terms. However, variance analyses for the interaction of clone and the segment type ($P = 0.01$) and for the interaction of clone, BA and the segment type ($P < 0.05$) were both significant. The difference in height increment between bottom and top segments was also significant ($P = 0.002$).

The presence of hormones in the growth media made significant differences on shoot growth. NAA had a significant promotory effect on growth ($P < 0.03$). BA, however had a significant inhibitory effect on growth ($P < 0.001$).

Analysis of variance for height increment

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Clone	7	252.42	252.42	36.06	1.50	0.182
NA	1	148.78	120.24	120.24	5.02	0.029
BA	1	1378.12	1177.50	1177.50	49.12	0.000
Type	1	435.12	248.57	248.57	10.37	0.002
Clone*NA	7	95.43	95.43	13.63	0.57	0.779
Clone*BA	7	193.92	193.92	27.70	1.16	0.341
Clone*Type	7	491.92	491.92	70.27	2.93	0.010
NA*BA	1	19.53	32.19	32.19	1.34	0.251
NA*Type	1	1.53	0.43	0.43	0.02	0.894
BA*Type	1	3.12	10.71	10.71	0.45	0.506
Clone*NA*BA	7	163.18	163.18	23.31	0.97	0.459
Clone*NA*Type	7	28.68	28.68	4.10	0.17	0.990
Clone*BA*Type	7	376.58	376.58	53.80	2.24	0.042
NA*BA*Type	1	63.28	22.53	22.53	0.94	0.336
Clone*NA*BA*Type	7	247.93	247.93	35.42	1.48	0.191
Error	64	1534.33	1534.33	23.97		
Total	127	5433.88				

Table 6.5 Effect of hormones on height increment on bottom and top segments.

It can also be seen from the table that there were no significant interactions between the hormones and the clones, and between the hormones and the segment types (bottom/top). The interaction of all the major factors (clone, NAA, BA and bottom/top) showed no significant difference on height increment. There was no bud outgrowth after transfer to hormone free medium.

6.2.3 Discussion

The results indicated that promotion of bud initiation by BA was highly significant (Tables 6.2 and 6.3). However, NAA did not promote bud initiation. The interaction between BA and NAA had no significant effect on bud initiation, compared to BA above, indicating that the two hormones had independent action even when they were present in the same medium.

The promotive effect of BA on bud proliferation has been reported for many different species. Arello and Pinto (1993) found out that when nodal segments of 45-day old *Kielmeyera coriacea in vitro* plants were cultured on MS medium supplemented with various combinations of BA and NAA, the highest shoot multiplication (6.3 shoots/explant) was achieved with 5.0 mg BA and 0.1 mg NAA/ℓ. Similarly, Dittmar (1991) reported that the culture of axillary buds and nodal segments of birch (*Betula pendula*) on WPM supplemented with 1 mg/ℓ BA and 0.05 mg/ℓ NAA gave the highest bud induction. In this work with *A. gracilior*, 2 mg/ℓ BA in MS medium promoted bud initiation in virtually every leaf axil.

In this study, the initiated buds failed to grow further even when they were transferred to hormone free medium. There are many reports that initiated buds can grow normally when the effect of the hormones is removed by transfer to hormone free medium. Buds were induced on a modified MS medium supplemented with BA and IBA, from cotyledons of 7-day-old Norway spruce, and then the transfer to hormone free medium stimulated bud growth (Krogstrup, 1989). This might indicate that BA is inhibitory to bud outgrowth although it has been proved that it was stimulatory to bud induction.

One might argue that the inhibitory effect of BA could depend on the concentration of this hormone and/or exposure time of the explants to the hormone.

A high concentration or long period of treatment with the hormone might increase the inhibitory effect even after the transfer of the initiated buds to hormone free medium. According to Salajova (1993), the development of induced buds occurred after transfer of explants to hormone-free medium and was affected by such factors as the BA concentration and exposure time. Cytokinin is often more effective in bud induction if applied in high concentration for a short period of time (pulse) rather than in low concentration in the medium for longer periods (Bonga and Aderkas, 1992). They further suggested that it is generally advisable to use suboptimal concentration of BA for shoot induction. Pérez-Bermúdez and Sommer (1987) in Bonga and Aderkas (1992) pointed out that BA concentrations that are optimal for initiation often cause poor subsequent shoot development

Some researchers claim that the addition of activated charcoal could improve the initiation and development of shoots. In cotyledonary explants of *Pinus canariensis*, bud development was enhanced by the elimination of phytohormones, a reduction of mineral salts and sucrose, and the inclusion of activated charcoal in the medium (Martinez-Pulido *et al.*, 1990).

The data presented here suggests that bud initiation occurs over a wide range of clones and that there was no significant difference between clones (Table 6.3).

A few bottom segments of BA treated cultures swelled particularly in the lower region of the stem close to the medium. In these cultures, bud initiation occurred not only in the leaf axils but also widely on the stem tissues (Figure 6.2). Eventually, these cultures appeared to have shown adventitious bud development rather than initiation of axillary buds. This result is in agreement with a report by Fouret *et al.* (1988) that shoot cultures of *Sequoia sempervirens* resulted in adventitious bud formation on the needles remaining on the stem stumps, and plants derived from these adventitious buds were orthotropic, whereas plants obtained by other protocols were plagiotropic.

In *A. gracilior* BA initiated more buds in bottom segments than in apical segments. It is possible that chemical compounds produced by the apical meristem might be suppressing the initiation of buds.

The effect of BA, NAA and BA*NAA interactions on shoot height increment was analysed (Table 6.4). It was calculated that BA significantly inhibited height growth compared to the control. It can also be seen from the same table that NAA enhanced height growth significantly compared to the control. The difference between segment type in stem elongation in the presence of NAA or BA was not significant (Table 6.5). In the interaction of BA and NAA, height growth was inhibited significantly by BA, regardless of the presence of NAA. This demonstrates that NAA had less promotory effect compared to the inhibitory effect of BA. The overall result of the interaction was significantly different to inhibition by BA alone.

The height growth inhibitory effect of BA and the growth stimulatory effect of NAA was independent of clone. Variation between clones as far as growth was concerned was not significant (Table 6.4). However, there was a significant interaction between clone, BA and type of explant.

There is some evidence that stem growth can be affected by cytokinins. Schwarz *et al.* (1988) in (Bonga and Aderkas, 1992) indicated that stem elongation in adventitious shoots of *Pinus strobus* was reduced by high concentration of BA in the medium during shoot induction, similar to the situation found in *A. gracilior*. In this work, NAA significantly stimulated stem elongation. However, it has been reported by other researchers that some auxins inhibited the growth of axillary shoots. According to Fouret *et al.* (1988) elongation in axillary shoot cultures of 500-year-old *Sequoia sempervirens* was inhibited by auxin and cytokinin, even at low concentrations.

6.2.4 Conclusions and Recommendations

1. Bud initiation was successful in this experiment.
2. Subsequent development of the initiated buds was inhibited. Bud outgrowth was not achieved in spite of transfer of the cultures to hormone free medium. This requires further investigation, looking particularly at lower concentrations of BA and the effect of pulse treatment.

6.3 Experiment 3: Rooting

6.3.1 Rooting with growth regulators

6.3.1.1 Materials and Methods

One hundred and nineteen shoot cultures that were actively growing with green stems and well extended green leaves were selected from a population of 17 mixed clones. The mixed clones had been maintained on MS hormone free medium for about two years and had been subcultured numerous times. Each of the selected cultures was a shoot with dominant apex. The cultures were treated for varying times on hormone containing medium before transfer to hormone free medium.

The cultures were randomised into seven groups of 17 explants. The seven groups of cultures were randomised into seven treatment periods: 0, 1, 2, 3, 4, 5 and 6 weeks. The cultures were grown on MS medium containing 5.37 µM NAA plus 4.90 µM IBA, i.e. 1 mg/ℓ of each (Appendix 6.2). The basal end of each culture was excised and the explants were transferred to the treatment media under sterile conditions. The experiment was rerandomised for incubation to avoid position effect. Treated cultures were labelled to identify hormone containing, number of weeks in hormone medium and position number. Clonal origin was ignored as a mixture of clones were used for the experiment. The cultures were incubated at 81 µmol photons m^{-2} s^{-1} at 20 °C.

Each group of cultures were transferred to basal medium after the required period of treatment. Assessments were made at weekly intervals for stem elongation, callus development and/or rooting for a period of 13 weeks.

6.3.1.2 Results

Two percent of the control cultures was rooted while none of the cultures in hormone containing media was rooted and therefore it was impossible to analyse the data.

(a) In general, shoot elongation decreased as the number of weeks the explants were exposed to the hormones in the medium increased (Figure 6.4). Analysis of

variance showed height increment between treatment weeks was significant (Table 6.6).

Analysis of variance for height increment

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Week	6	1093.75	1093.75	182.29	14.54	0.000
Error	112	1404.12	1404.12	12.54		
Total	118	2497.87				

Table 6.6 Analysis of variance for height increment differences between different treatment periods after 13 weeks.

(b) Callus diameter was analysed based on the number of weeks the explants were in culture.

Analysis of variance for callus diameter

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Week	6	403.97	403.97	67.33	20.03	0.000
Error	112	376.47	376.47	3.36		
Total	118	780.44				

Table 6.7 Analysis of variance for callus diameter growth differences between different treatment periods after 13 weeks.

Callus development at the base of shoots not treated with the hormone containing medium was poor. There was an increase in the diameter of the callus that developed at the base of treated shoots with an increased duration of treatment (Figure 6.5). Statistical analysis demonstrated that this increase in callus development was highly significant (Table 6.7).

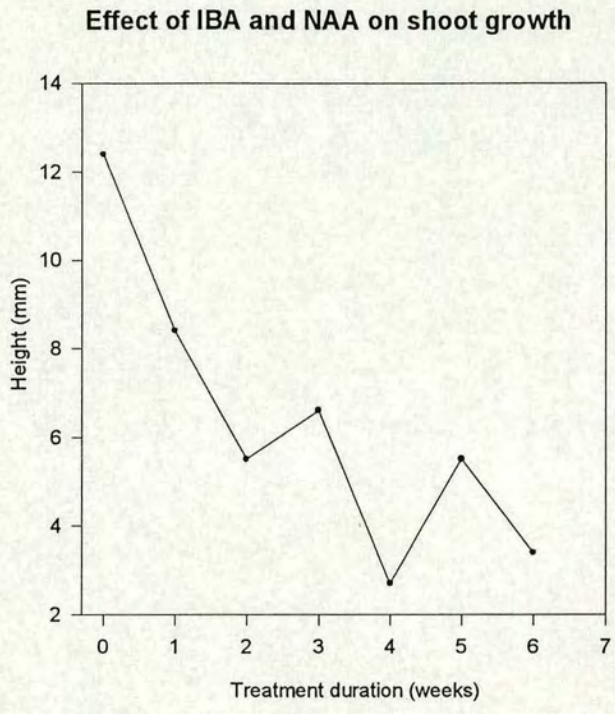


Figure 6.4 The effect of IBA plus NAA, applied for different durations, on shoot growth *in vitro* after 13 weeks (sd 1.21).

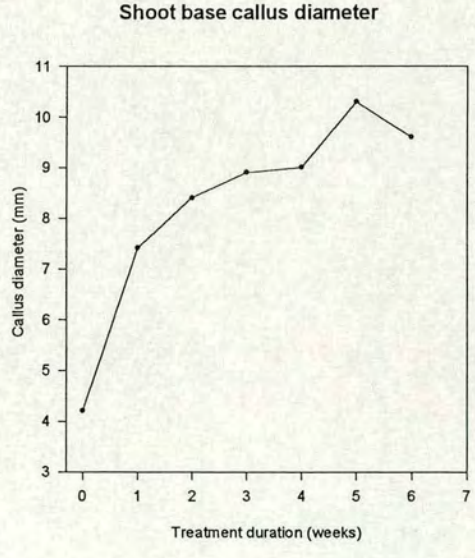


Figure 6.5 The effect of IBA plus NAA, applied for different durations, on callus diameter growth differences after 13 weeks (sd 0.63).

6.3.1.3 Discussion

This experiment was designed to root the shoots *in vitro* with the auxins IBA plus NAA in combination. The literature on rooting of *in vitro* shoots with these growth regulators was surveyed and the concentrations selected were considered to be the best option.

Treated shoots did not root either during the period of 13 weeks treatment or after they were transferred to hormone-free medium. Since none of the treated shoots rooted, the data for the spontaneously rooted control shoots were not analysed. Several other experiments have indicated that there was a spontaneous rooting of this species *in vitro* to about 35-40% whereas in this experiment, spontaneous rooting was very low at 2%.

The reasons for failure of rooting with the concentrations of IBA plus NAA in combination used cannot be explored in this experiment. An answer can only be obtained by doing further experiments using various hormone concentrations and types of media. The literature regarding rooting conditions of other species suggests that some species root better with lower concentrations than those used in this experiment while others needed more concentrated rooting hormones.

Treatment duration could also be another possible area of investigation. Some species need just a pulse treatment rather than a longer period of exposure to rooting hormones. Shoots of *Quercus suber* have been successfully rooted following dipping in concentrated IBA solution (Romano *et al.*, 1992) and also shoots of *Wrightia tomentosa* in 100 mg pre-autoclaved IBA/litre for 15 minutes (Purohit *et al.*, 1994). Furthermore, pulse treatment of elongated *Pinus halepensis* with 1 mM IBA for six hours gave 90% rooting (Lambardi *et al.*, 1991).

There have been reports that changing the type of medium used for rooting has a significant effect. Some researchers use WPM supplemented with relatively lower concentrations of IBA plus NAA to root some *in vitro* propagated species. According to Bueno *et al.* (1992) induced buds of Populus alba '*Siberia extremena*' subcultured in modified MS medium rooted in WPM plus 0.2 mg/litre IBA with 95% success. Similarly, Perez-Parron *et al.* (1994) pointed out that *Fraxinus augustifolia* rooted on WPM supplemented with 0.98-4.9 mM IBA.

It has also been suggested that other components of the medium have a significant effect on rooting, e.g. high sucrose concentrations improved rooting of *Quercus suber* cultures (Manzanera and Pardos, 1990).

Height increment and callus diameter were recorded and eventually analysed at the end of the 13 weeks the explants were in culture. Analysis of variance indicated that there was a significant difference of height increment between weeks of shoot treatment (Table 6.6). It can also be seen from Figure 6.4 that height increment decreases with an increase in the number of weeks of treatment. The two hormones resulted in forming callus at the base of the explants and also inhibited shoot elongation. It could be noted (Section 6.2) that 0.5 μM NAA promoted shoot elongation. There could therefore be two reasons for shoot inhibition in this experiment. It could be that the concentration of NAA might be too high. A second reason might be that IBA itself is inhibitory to extension growth or that the combination of the two compounds at relatively high concentrations was inhibitory. There was a sharp decrease in height increment between zero and two weeks of hormone treatment, but the subsequent decrease was less with increasing duration of treatment.

Callus development has also been analysed. There is a significant difference between weeks of treatment in callus diameter (Table 6.7). It can be seen (Figure 6.5) that there was a sharp increase in callus diameter between the zero and the one week treatment. The rate of increase in callus diameter was less beyond one week of treatment. The general trend of callus development was that it increased with an increase in the number of weeks of treatment, and that the rate of increase decreased at longer periods of treatment.

6.3.1.4 Conclusions and Recommendations

1. This species did not root with the selected concentration of the two rooting hormones.
2. It is probable that a series of experiments needs to be designed to optimise the concentrations of the rooting hormones.

3. The medium used in this experiment was MS. It is possible that other media such as WPM or modification of the media contents, such as sucrose concentration might improve rooting.

6.3.2 Rooting with aqueous leachate of seed coat of its own species

6.3.2.1 Materials and Methods

Two hundred grams of A3 seed coat were soaked in 2 litres of distilled water for 48 h at 4 °C. The solution was filtered to remove the solid material. This concentration was taken to be 100% leachate. A dilute concentration of 25% was prepared by taking 250 ml of 100% and mixing with 750 ml distilled water. The control contained no leachate.

Each concentration was stirred with 30 g of sucrose and 4.4 g of MS salt (macro and micronutrients, and vitamins as described by Muroshige and Skoog (1962)) per litre. The pH of the solution was adjusted to 5.5. The solution was heated to boiling and 10 g of agar were added. Twenty ml of the media was dispensed into each 15 cm soda-glass test tube. The solution was autoclaved for 20 minutes at 121° and the media were subsequently stored at 2 °C before transfer of the shoots.

Fifty-four shoot cultures that were actively growing with green stems and well extended green leaves were selected from the population of mixed clones. The mixed clones had been maintained on MS hormone-free basal medium for about two years and had been subcultured numerous times. Eighteen cultures were inoculated into each medium and randomly incubated at 81 μmol m^{-2} s^{-1} at 20 °C. Assessments were made for rooting and height increment.

6.3.2.2 Results

It was found that after 65 days of incubation, rooted shoots were present in all treatments (Table 6.8). However, whereas 22% of the shoots were rooted in 0% leachate medium more than 60% were rooted in the 25% leachate medium. Analysis of variance of the arcsine transformed rooting percentages showed that the differences in rooting were just significant (Table 6.9).

Summary of rooted and non-rooted

Conc %	Rooted	Non-rooted	Rooting %
0	4	14	22.2
25	11	7	61.1
100	6	12	33.3

Table 6.8 Number of rooted and non-rooted shoots in the three concentrations of the leachate after 9 weeks.

(i) Analysis of variance for transformed data of rooting

Source	DF	SS	MS	F	P
Concentration	2	0.08	0.04	3.23	0.048
Error	51	0.65	0.01		
Total	53	0.73			

Table 6.9 Analysis of variance on the effect of leachate concentration on rooting.

(ii) Analysis of variance for shoot elongation

Source	DF	SS	MS	F	P
Concentration	2	75.26	37.63	2.47	0.095
Error	51	777.72	15.25		
Total	53	852.98			

Table 6.10 Analysis of variance for height increment (mm) between concentrations of the leachate (0, 25 and 100%) after 9 weeks.

The mean height increment measured for the 0, 25 and 100% leachate concentration were 9.39, 8.06 and 6.50 respectively. Analysis of the data demonstrated that there were no overall significant differences in growth between the different treatments (Table 6.10). However, there was a significant difference between the 0 and 100% treatments (LSD 2.60). Note that tests on selected

comparisons are legitimate provided the selection is based on *a priori* grounds, and not on inspection of the results.

6.3.2.3 Discussion

This experiment was undertaken after an observation of inhibition of this leachate on the germination of wheat seed (Chapter 2). They are substances in the leachate that make it physiologically active and this experiment was established to investigate their effect on shoot elongation and development.

Analysis of variance indicated that there was no significant differences between height increments of the three concentrations used (Table 6.10). However, LSD (2.60) between mean height increment of zero and 100% leachate concentrations was calculated to be significant. The trend of effect was that shoot growth was inhibited with an increase in leachate concentration. One of the supportive experiments proved that when this leachate was autoclaved to 40 minutes, it was also found to have inhibited germination of wheat showing that it is not heat labile, however, unlike in this experiment, it was not boiled before it was autoclaved. The inhibitory compound from the seed coat of this species described in Chapter 2 was Nagilactone D, found to be allelopathic to germinating wheat seed. The compound(s) which were responsible for the inhibition of growth of shoot cultures in concentrated leachates, particularly at 100% concentration, are not known.

Those chemicals which appear to have some effect on its own seedlings could have some kind of advantage to survival. In field conditions, inhibitors control germination of its own seeds until the condition for growth is favourable. The inhibitors function predominantly as germination controls which regulate densities within the population (intraspecific inhibition) and allow germination only after a sufficient amount of rainfall (Friedman *et al.*, 1982). The inhibitors can contribute to some ecological implications in that the seeds stay quiescent, enriching the seed bank and, as for many other species, serving as a reserve for renewing the population after years of shortage of rainfall (Friedman *et al.*, 1982). On the other hand, they pointed out that the inhibitors, with their slow efflux, could account for decimation of species other than its own and thus reduce interspecific competition.

The seed coat could contain such complex structures which might have contributed to the evolutionary sequence of the species. The fact that the leachate had little inhibition on its own seedlings compared to complete inhibition of wheat seed could show that in field conditions, the inhibitory effect of these chemicals could only be conditional.

It is interesting to note that the crude leachate extracted from the seed coat had a significant effect on root initiation at the base of the cultures with the concentration of 25% promoting rooting to 60%. The effect of leachate on rooting was investigated further and reported below (Section 6.3.3).

6.3.2.4 Conclusions

The leachate had an inhibitory effect on the germination of wheat seeds and also on the extension of *A. gracilior* shoot cultures. However, there was a promotory effect on the rooting of the cultures.

6.3.3 Rooting with aqueous leachate of seed coat of its own species

6.3.3.1 Materials and Methods

Two hundred grams of the seed coats, a mixture of batches A2 and A3, were soaked in two litres of distilled water. The mixture was filtered twice to remove solid material and the filtrate was diluted to various concentrations. The original leachate was taken as 100% concentration. Lower concentrations were prepared from the original concentration as follows: The 100% leachate was diluted with distilled water to give concentrations of 10, 20, 30, 40 and 50% with distilled water a control. The media were prepared as described previously (Section 6.3.2).

Ninety eight shoots of the clone mixture were randomly divided into seven groups of 14 each. All shoots were trimmed at the base and randomly transferred to the seven media using standard *in vitro* techniques. Each shoot was randomly located in the growth room so as to eliminate position effect. Assessments were done on a weekly basis for rooting and shoot elongation.

6.3.3.2 Results

(i) *Rooting*

Twenty-nine percent of the shoots used in the experiment were rooted over 62 days of the culture period (Table 6.11). Shoots in the leachate concentration of 10 and 100% rooted to 43% each which was relatively the best. The poorest rooted cultures were those in the concentrations of 0 and 50% with 14% each. The rooted cultures in the 100% concentration showed the highest number of roots per shoot with an average of four. The rooted cultures in the 10% concentration developed an average of two roots per shoot. The remaining concentrations showed only one root per shoot in the rooted cultures. The Chi-square test was used to check whether rooting was significant compared with the unrooted cultures. The Chi-square test demonstrated that there were no significant differences in rooting between the various media used (Chi-square = 14.4, P = 0.28).

(ii) *Shoot extension*

There was a general trend to a reduction in the measured height increment with an increase in the concentration of the leachate used in the medium (Figure 6.6). However, the medium at a concentration of 10% caused a large reduction in growth compared to the other dilute concentrations.

The highest shoot extension was observed in the cultures with the concentration of 0% (control), the increment being 5.5 mm over 62 days of the culture period. The least increment was seen in the concentration of 100% leachate which was 3.4 mm. Regression analysis showed that there was a significant difference between leachate concentrations, F = 5.63, P = 0.02 (Table 6.12).

Chi-square test for rooting

% Concentration	No. Callus	No. Root	No Change	Total No.
0	10 8.57	2 4.0	2 1.43	14
10	6 8.57	6 4.0	2 1.43	14
20	10 8.57	2 4.0	2 1.43	14
30	9 8.57	5 4.0	0 1.43	14
40	9 8.57	5 4.0	0 1.43	14
50	11 8.57	2 4.0	1 1.43	14
100	5 8.57	6 4.0	3 1.43	14
Total	60	28	10	98

Table 6.11 Chi-square for rooting of the shoot in leachate media after 9 weeks. Chi-square = 14.4, df = 12, P = 0.28; (Table value = 21). The expected values for the column are callus = 8.57, root = 4 and no change = 1.43. The numbers in the table are the number of shoots that were observed and expected to be callused, rooted or shown no change against each leachate concentration.

Regression analysis for height increment

Source	DF	SS	MS	F	P
Regression	1	32.371	32.371	5.63	0.020
Error	96	552.119	5.751		
Total	97	584.490			

Table 6.12 Regression analysis for shoot extension between different leachate concentrations after 9 weeks in culture. The regression equation is increment = 5.18 - 0.019 concentration.

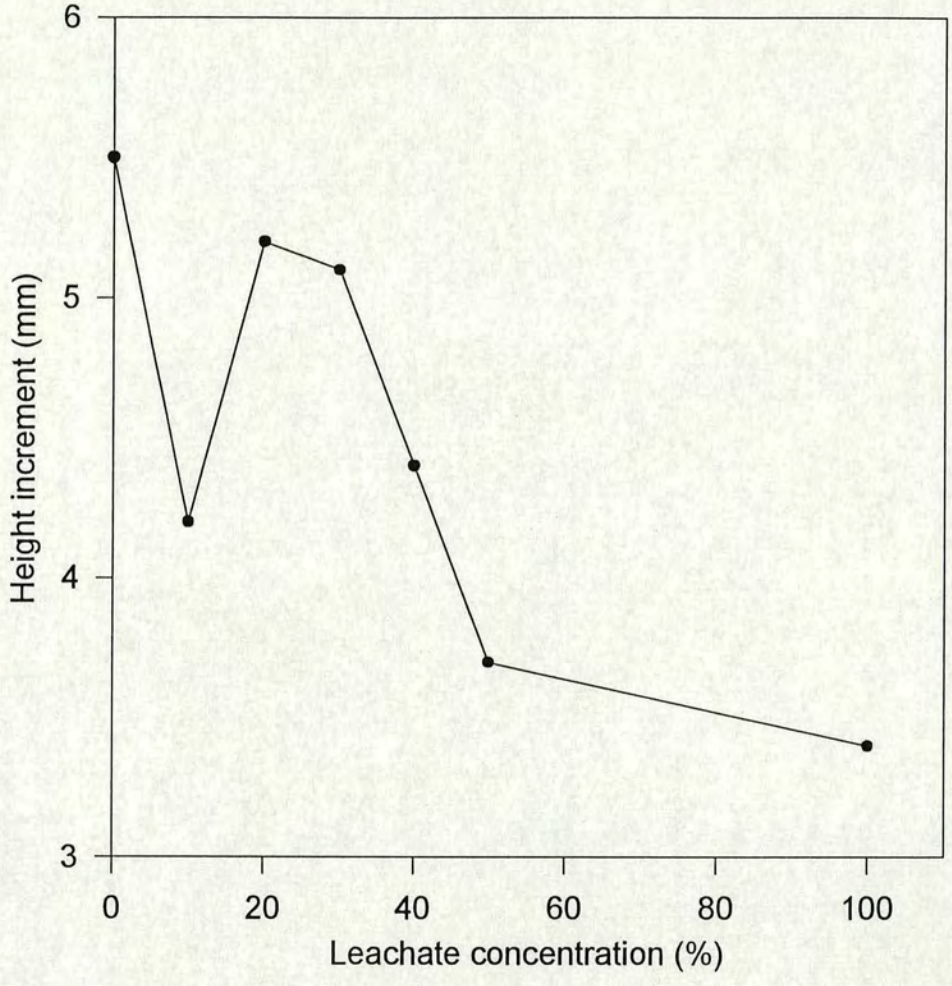


Figure 6.6 Shoot height increment in leachate media after 9 weeks of culture period.

(iii) *Shoot condition*

The condition of the shoots was evaluated subjectively by giving scores as follows: 1. Bad - little or no extension with all tissues mostly brown; 2. Poor - little extension with tissue mostly yellow; 3. Fair - some extension with mostly green tissues but with some yellowing; 4. Good - well extending with green stems and well extended green leaves (Table 6.13).

	Score				
Conc %	**1**	**2**	**3**	**4**	**Missing**
0		1	1	11	1
10		2	3	9	
20		1	3	10	
30		2	2	10	
40			3	11	
50		2	2	10	
100		2	3	7	2

Table 6.13 Scoring of shoot condition after 9 weeks in culture.

Most of the shoots were in good condition. There were no significant differences between leachate concentrations on scoring the defined conditions (bad, poor, fair or good), P = 0.81. Siegel (1956), Kruskal-Wallis test, describes this type of non-parametric analysis.

Kruskal-Wallis Test

Conc %	No. Observation	Mean Score
0	13	53.9
10	14	44.4
20	14	48.4
30	14	47.4
40	14	52.4
50	14	47.4
100	12	41.0
Overall	95	48.0

Table 6.14 Kruskal-Wallis test applied to shoot condition scores.

H = 1.87 d.f. = 6 P = 0.93
H = 2.98 d.f. = 6 P = 0.81 (adj. for ties)

6.3.3.3 Discussion

Results indicated that there was no significant difference between leachate concentrations on rooting (see Table 6.11) and the effects produced by the leachate concentrations on rooting were not in any sort of sequence. The number of roots produced by individual shoots was highest in the 100% concentration (data not shown).

There was no significant difference between effects of concentration on shoot elongation (see Table 6.12). Generally, height increment decreased as the concentration of the leachate increased. However, height increment for the leachate concentration of 10% did not agree with this generalisation. It could be possible that this happened because of experimental error. It seems that there was stronger inhibition in this leachate than the leachate in the previous experiment (6.3.2).

The leachate of this experiment was prepared from aqueous solutions of seed coats of two batches. In comparing the two leachate experiments, both leachates had less inhibition to its own species compared to complete inhibition to germinating wheat seed. However, the leachate of this experiment, which was obtained from two seed batches, inhibited relatively more than the single batch seed leachate in the previous experiment. This might indicate that some seed coats might contain more inhibitory elements than the other depending on the environmental condition of the mother tree during seed development.

6.3.3.4 Conclusions

1. The fact that the interaction of leachate from two seed batches inhibited root formation and shoot elongation more than the single leachate from A3 seed batch might be that the leachate from A2 could contain more inhibitors or there might be a synergistic effect of the two leachate on the explants.
2. The second possibility for the difference could be that the explants used in this experiment were not physically large enough to produce roots.
3. More research is required to investigate clearly leachate effects from a single seed lot and the interaction of more leachates on shoots.

6.4 *Ex vitro* rooting

6.4.1 Introduction

In this experiment, shoots multiplied under *in vitro* conditions were rooted *ex vitro* in soil. The main advantages of *ex vitro* over *in vitro* rooting are that root damage during transfer to soil is less likely, rooting rates are often higher and root quality is often better (Bonga and Aderkas, 1992).

6.4.2 Materials and Methods

The shoots were rooted in semi-transparent polycarbonate boxes (L = 12 cm, W = 9 cm, H = 7 cm). The rooting medium used was a mixture of standard compost (peat-grit-loam with microelement and frit) and coarse grit in equal proportions. Frit is a commercial preparation of trace elements and chelated iron. The rooting medium was moistened before use and the boxes were filled to a depth of 3 cm. Sixteen boxes were prepared.

In vitro growing shoots subcultured numerous times on hormone-free medium, approximately 2.5 cm, 2.5 years old were basically trimmed and washed in tap water to remove all traces of agar. The shoots were either basically dipped or not dipped in commercial rooting powder, Seradix No 2 (May and Baker Ltd) and struck into the compost.

The boxes were closed with a semi-transparent polycarbonate lid that was sealed with adhesive PVC tape. The rooting containers were incubated in a growth room at 20 °C with a 16 hour day under 'high light' (45.6 µmol photons m^{-2} s^{-1}) or 'low light' (7.9 µmol photons m^{-2} s^{-1}). The photon flux densities were measured at the same distance from the light source as the lid of the rooting containers. It was not possible to measure the photon flux density within the rooting containers.

There were 6 shoots per box (Figure 6.7) and 4 boxes of hormone treated or not hormone treated per light treatment. The shoots were inserted in the various treatments at random. The boxes were selected at random for light treatment. The bases of the shoots were examined at weekly intervals for signs of rooting. Dead

shoots were defined as those which had lost turgor and were brown to black in colour and were removed at the time of examination.

6.4.3 Results

6.4.3.1 Survival

	Days										
Treatment	6	13	21	30	37	48	54	73	82	90	97
H^+L^+	24	24	24	24	23	20	20	19	17	16	16
H^+L^-	24	24	23	19	16	9	9	6	5	4	4
H^-L^+	24	24	24	24	22	20	20	20	20	20	19
H^-L^-	24	24	21	18	14	14	14	11	11	9	9

Table 6.15 Number of shoots surviving over the incubation period of 97 days. (H^+ - hormone treated, H^- - not hormone treated, L^+ - high light, L^- - low light).

All shoots survived at least for 13 days while 50% of the shoots survived for 97 days (Table 6.15). Percentage survival for 97 days of the treatments H^+L^+, H^+L^-, H^-L^+ and H^-L^- were 67, 13, 82 and 38 respectively. A Chi square test ($\chi^2 = 28.33$, $P < 0.001$) showed that there was a highly significant difference between treatments. A further Chi square test between high and low light indicated that there was a highly significant difference ($\chi^2 = 24$, $P < 0.001$). Similarly, the difference between H^+ and H^- on shoot survival was significant ($\chi^2 = 4.16$, $P < 0.05$).

Figure 6.7 Micropropagated shoots of *A. gracilior* in the *ex vitro* rooting. The lid has been removed (bar: 2 cm).

6.4.3.2 Callusing

	Days										
Treatment	6	13	21	30	37	48	54	73	82	90	97
H^+L^+	0	1	4	11	12	13	15	16	16	16	16
H^+L^-	0	3	4	4	4	4	5	5	5	5	5
H^-L^+	0	1	5	13	15	15	16	16	16	17	17
H^-L^-	0	0	5	5	5	5	6	6	6	6	7

Table 6.16 Number of shoots developing callus over the incubation period of 97 days. Shoots were counted as callused for the duration of the experiment even if the shoot subsequently died.

There was no callus development in any culture in the first 6 days (Table 6.16). Callus development was observed after 13 days: the percentage occurrence for H^+L^+, H^+L^-, H^-L^+ and H^-L^- were 4, 13, 4 and 0 respectively. Cultures treated with H^+L^+ and H^-L^+ had sharp increases of callus number between 21 and 30 days. In general, all treatments showed an increase in the number of shoots callused between 13 and 54 days (Figure 6.8). The percentage callus development after 97 days for H^+L^+, H^+L^-, H^-L^+ and H^-L^- were 67, 21, 71 and 29 respectively.

A Chi square test showed that there was a highly significant difference between treatments ($\chi^2 = 18.87$, $P < 0.001$). A further test was carried out and the difference between H^+ and H^- was not significant ($\chi^2 = 0.39$, $P > 0.50$). However, the difference between L^+ and L^- was highly significant ($\chi^2 = 18.45$, $P < 0.001$). The interaction between the hormone and the light was not significant ($\chi^2 = 0.04$, $P > 0.50$).

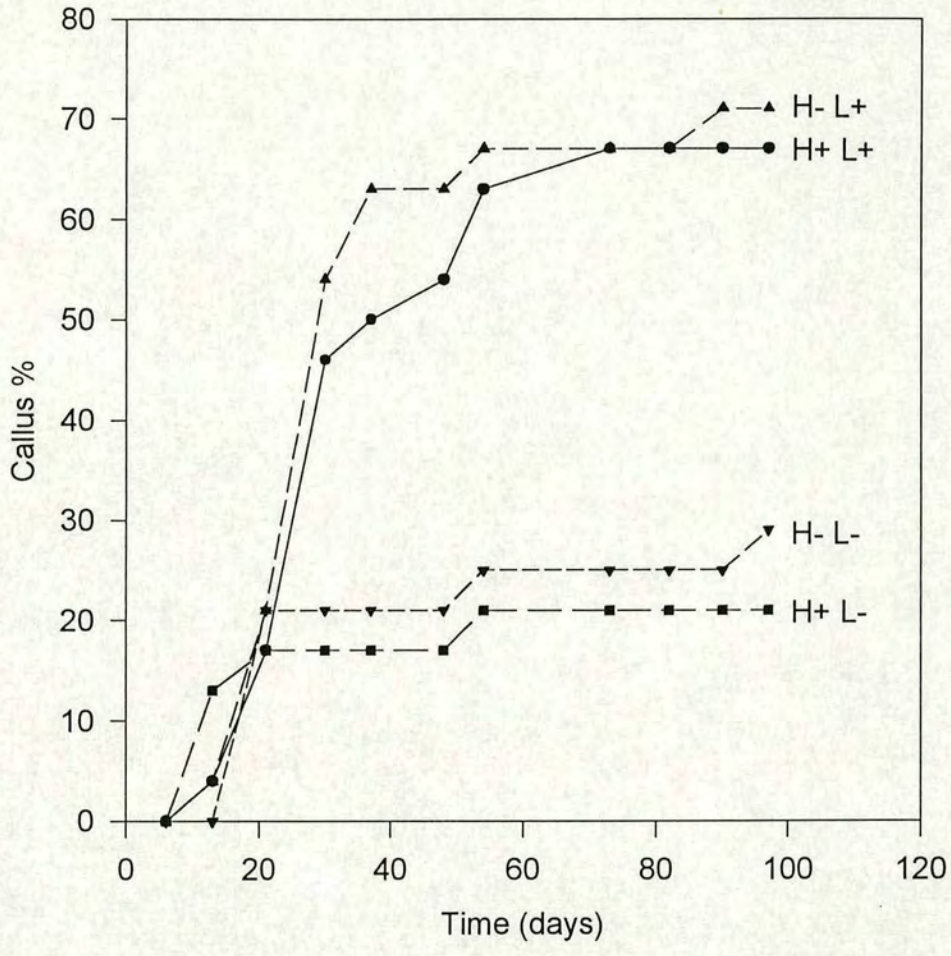


Figure 6.8 Percentage callusing of shoot bases over 97 days.

6.4.3.3 Rooting

	Days										
Treatment	6	13	21	30	37	48	54	73	82	90	97
H^+L^+	0	0	0	0	0	0	2	3	4	5	6
H^+L^-	0	0	0	0	0	0	1	1	1	1	1
H^-L^+	0	0	0	0	0	9	9	9	9	9	10
H^-L^-	0	0	0	0	0	1	1	1	1	1	1

Table 6.17 Number of shoots rooting over the incubation period of 97 days.

The first rooted shoots were observed after 48 days (Table 6.17). There was a linear increase in rooting in H^+L^+ between 48 and 97 days whereas in H^-L^+ there was initially rapid rooting followed by a slow subsequent increase with time (Figure 6.9). Rooting was very poor in the other two treatments. The percentage of root formation after 97 days for H^+L^+, H^+L^-, H^-L^+ and H^-L^- were 25, 4, 42 and 4 respectively.

A Chi square test showed that there was a significant difference between treatments ($\chi^2 = 15.59$, $P < 0.01$). There was no significant difference between H^+ and H^- ($\chi^2 = 1.09$, $P > 0.10$). However, the difference between L^+ and L^- was calculated to be highly significant ($\chi^2 = 13.40$, $P < 0.001$). There was no interaction between the light and the hormone ($\chi^2 = 1.09$, $P > 0.10$).

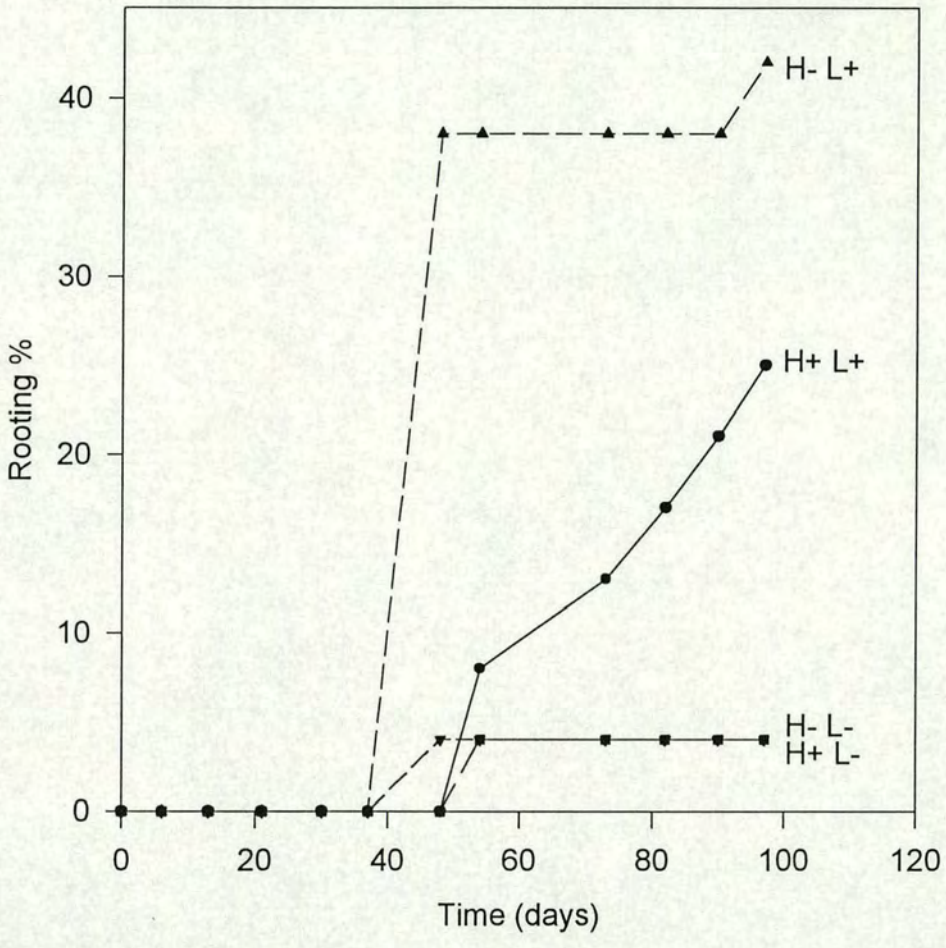


Figure 6.9 Percentage rooting of shoots over 97 days.

6.4.3.4 Shoot growth rate

Death and removal of shoots from the experiment presented a number of problems when attempting to analyse the growth data. It was therefore not possible to calculate overall growth rate means. However, it was possible to calculate the rate of growth per day for each shoot for as long as it survived. The daily growth rate data was analysed by analysis of variance.

Source	DF	SS	MS	F	P
Light	1	0.050949	0.050949	12.79	0.001
Hormone	1	0.005824	0.005824	1.46	0.23
Light*Hormone	1	0.001428	0.001428	0.36	0.551
Error	92	0.366497	0.003984	0.36	0.551

Table 6.18 Analysis of variance for daily growth rate

It can be seen from Table 6.18 that the effect of light on growth was highly significant. However, the effect of hormone or the light*hormone interaction was not significant.

The shoots treated with L^+H^+ showed the highest growth rate, although they were not significantly different from the shoots treated with L^+H^- (Table 6.19). The shoots treated with L^-H^- grew poorly. There was a significant difference between L^+ and L^- while there was no significant difference between H^+ and H^-.

	L^+	L^-
H^+	0.041^{a}	0.002^{bc}
H^-	0.033^{ab}	-0.021^{cd}

Table 6.19 The mean growth rate (mm per day). Means with different superscripts are significantly different from each other.

6.4.4 Discussion

The results indicate that light was the major effect on the whole process of rooting. It is possible that under low level of light, some processes like photosynthetic pathways and nitrate-reduction activities were affected. In virtually all types of plant growth, light is, of course, of major importance, since it is the source of energy, and in rooting leafy cuttings, the products of photosynthesis are important for root initiation and growth (Hartmann and Kester, 1975). The low level of light used in this experiment could have sufficiently affected the process to result in death of a large proportion of plants and a large number of shoots were dead by 48 days in the low light treatment.

In the high light, normal development of callus occurred by the time the majority of shoots died in the low light. Callusing in the low light was much lower at 37 days although there were bases that could have developed calluses. It is possible that basal swellings and callusing can increase the surface area of the shoot so that water absorption and plant access to nutrients were improved. Hence, many of the shoots that developed calluses subsequently rooted in the high light while those in the low light died possibly because of the poor condition of the plants.

Rooting hormone treatment had no significant effect on callusing and on subsequent rooting. However, the hormone tended to inhibit rooting compared to non-treated shoots.

Shoot extension was also significantly affected by the light level. There was normal growth and development of the plants in the high light while those in the low light performed poorly. There was no significant effect of rooting hormone on shoot extension although treated shoots appeared to grow better than the non-treated shoots.

It is interesting to note that the level of rooting achieved here (40%) is comparable to the spontaneous rooting observed in several previous experiments. However it is lower than the 60% observed in one of the leachate experiments (Section 6.3.2). This would seem to indicate that there is the potential for improvement in *ex vitro* rooting and that the conditions used in this experiment were far from optimal.

6.4.5 Conclusions and Recommendations

Light is an important factor which influences *ex vitro* rooting in *A. gracilior*. The optimum level of light should be investigated in future research.

6.5 Transfer of rooted shoots to soil

6.5.1 Introduction

Plant multiplication *in vitro* is carried out usually in agar media where the relative humidity is close to 100%. A large number of plants can be produced as it is possible to work with small shoots with the *in vitro* techniques. However, the propagated plants need to be put back into the natural environment for normal growth and development. When the required number of *in vitro* multiplied and rooted plants is obtained, they are transferred to soil and weaned for a certain period of time. This experiment aimed to transfer *in vitro* multiplied plants to soil condition through an acclimatisation process.

6.5.2 Materials and Methods

The potting compost was prepared by mixing 60% peat, 30% grit, 10% sterilised loam, osmocote slow release fertilizer and trace elements (Frit 253A).

The compost was moistened with just sufficient water so that it would be loose enough for good aeration. A plastic pot (13 × 13 × 15 cm) was filled with the compost near to the top.

Twenty-eight rooted shoots grown *in vitro* in leachate media of the experiment (6.3.3) for 72 days were transferred to the soil so that one rooted plant was planted in each pot. A rooted shoot was carefully taken out of the test tube by scooping out together with part of an agar to avoid root damage. The agar was completely washed off from the basal portion of the plant. A hole was made into the soil and the plant was inserted into it so that the root was completely covered with the soil. A transparent plastic cap (Stewart single pot propagator, diameter 95 mm, height 140 mm) was inverted on the soil above the plant in such a way that it created a humid environment around the shoot. Light was available to the plant and the space inside was enough for aeration and growth. The inverted cap was initially completely sealed

to avoid moisture shock in the initial stage. The plants were incubated in a growth room under a fluorescent light of 97 μmol photons m^{-2} s^{-1} for two weeks at 20 °C. They were transferred to a glasshouse where the position of the pot was randomised. The pots were arranged in rows of 4 × 7 in the glasshouse at a minimum temperature of 16 °C. The soil was watered twice a day.

The relative humidity around the shoots was reduced after four weeks by opening the ventilation holes in the plastic caps. The shoots were exposed to normal greenhouse conditions after a further two weeks by completely removing the plastic cap. The plants were transferred to the soil and weaned from the middle of August 1995 to early October 1995.

6.5.3 Results

All plants survived weaning. However, there was a subsequent loss of some plants in the greenhouse, particularly those along the edge of the table near to the glass wall.

Glass Wall Corner

X	X	X	X	A	A	A
A	A	A	A	A	A	A
A	A	A	A	X	X	A
A	A	A	A	A	A	A

Side near to middle

The survival of rooted plants of the weaning. Dead plants (X) and live plants (A).

6.5.4 Discussion and Conclusions

It is interesting to note that these *in vitro* plants successfully survived after they were put back into the natural environment. The weaning method used here was also found to be very useful and applicable since survival rate was 100%. However, care should always be taken during the weaning process to keep the plants away from any heat source in growth rooms or glasshouses as the plants are susceptible to heat stress.

CHAPTER 7

Somatic embryogenesis of *A. gracilior*

Introduction

Somatic embryogenesis is a process by which somatic cells develop into complete plants through stages characteristic of zygotic embryo development. Tissue cultures of various plant species grown on suitable media are capable of forming plantlets, which more or less repeat the developmental stages of zygotic embryos (Radojevic *et al.*, 1975). Unlike organogenesis, therefore, somatic embryogenesis can regenerate events in zygotic embryogenesis with the production of embryos having shoot and root apices or bipolarity (Tautorus *et al.*, 1991). Somatic embryos arise from single superficial cells of the embryonic clumps by a sequence of segmentation (Konar *et al.*, 1972; McWilliam *et al.*, 1974).

Somatic embryogenesis was first reported on a carrot plant in 1958 (Steward *et al.*, 1958). They described their work as "In the regenerated plants, the axis with shoot and root apices is completely established: cells drawn from the phloem of the storage carrot root and which have passed through many transfers in which they were reduced to the single cellular state, have developed into cell aggregates, which have, in turn, differentiated to form roots and, when transplanted, have given rise also to shoots and to a secondary thickened storage carrot root".

The technique has covered a huge range of species in many genera in the last three decades. Since the development of somatic embryogenesis in carrot tissue cultures, numerous angiosperm species have been regenerated *in vitro* by this method (Kendurkar *et al.*, 1995).

Somatic embryogenesis in woody species has recently been developing with the possibility of using the technique at a wider base in plant propagation methods. A key element in the application of the technique in somatic embryogenesis to woody perennials is the development of systems for the regeneration of plants from protoplasts, cells, tissues and organs (Tulecke and McGranahan, 1985).

Somatic embryogenesis in conifer species has developed in the last ten years. Somatic embryogenesis and plantlet regeneration was first reported in 1985 on *Picea*

abies for which zygotic embryos were used as explants (Hakman *et al.*, 1985) and since then, a great deal of progress has been made for conifer reforestation (Gupta *et al.*, 1993).

Various methods and culture procedures have been used so as to achieve considerable results in somatic embryogenesis. The phytohormones 2,4-D and BA have been ubiquitously used at various concentrations to both induce and maintain somatic embryos of most species (Tautorus *et al.*, 1991). The manipulation of physical, nutritional, and hormonal environment are important in this process.

The state of explant will to a large extent determine the success or failure of somatic embryogenesis. Tautorus *et al.* (1991) pointed out that a proper explant is critical in order to achieve successful induction of somatic embryogenesis. Various tissue parts of the same plant can show different responses to the induction of somatic embryogenesis. Similarly, the same plant at different stages of development can give different results as a response to somatic embryogenesis. Bonga and Aderkas (1992) showed that the more juvenile the plant material, the easier it is to induce somatic embryogenesis. Somatic embryogenesis in conifers (except in a few cases) has been almost exclusively confined to embryonic explants. Several researchers investigated a better response of early stage zygotic embryos to the induction of somatic embryogenesis. Precotyledonary zygotic embryos are best for induction of embryogenic tissues in *Pinus* species (Tautorus *et al.*, 1991) and the optimum explants which are responsive to somatic embryogenesis are zygotic embryos at precotyledonary stage of development in long leaf pine, *Pinus palustris* (Nagmani *et al.*, 1993). In the early stages of development, embryos and gametophytes could be treated together for the induction of somatic embryogenesis. Owens and Molder (1984) in Nagmani *et al.* (1993) reported that all of the long leaf pine embryogenic cultures initiated were from female gametophytes with intact zygotic embryos at early stages of development.

Various concentrations of growth substances, notably, 2,4-D and BA have been frequently used to induce somatic embryogenesis on MS or some other media. The mechanisms by which 2,4-D operates to achieve embryogenesis are not well understood. The effects of various concentrations and combinations of growth

regulators were tested and it was found that a combination of 2,4-D (10^{-5} M) and BA (10^{-6} M) was best in *Picea abies* (Hakman *et al.*, 1985). In some cases, 2,4-D might be required for a relatively short period of time. The 2,4-D is generally only required during the embryogenesis initiation phase, and in most cases the auxin has to be withdrawn once the embryos have appeared and in a few cases, 2,4-D and other auxins will inhibit somatic embryogenesis (Bonga and Aderkas, 1992).

Somatic embryos might appear directly from the explants or they are formed after a callus stage in the process. When callus is formed, it is not necessarily embryogenic, but will serve as the precursor tissue for further differentiation of embryogenic callus (Wann, 1989).

Many reports indicated that embryo maturation has been enhanced in the presence of ABA. Bonga and Aderkas (1992) reported that at the highest ABA concentration (30-40 mM), *Picea glauca* somatic embryos matured and germinated normally. Slow drying (at 95% RH) was as effective as ABA in obtaining normal maturation and germination of somatic embryos of *Picea abies* (Roberts *et al.*, 1990). Maturation of embryos of *Pinus caribaea* was achieved on full macronutrient concentrations rather than diluted mineral salts and by adding ABA to the medium (Laine and David, 1990). Most commonly, initiation, maintenance and maturation of conifer somatic embryos is reported on medium supplemented with 30-90 mM sucrose (Tautorus *et al.*, 1991).

Germination and conversion of oak (*Quercus robur*) somatic embryos into plantlets was achieved on WPM containing a reduced concentration of cytokinin and in *Tilia cordata* it was achieved on MS medium containing a low concentration of IBA, and plantlets of both species were successfully established in soil (Chalupa, 1990). Proliferating embryogenic cultures from conifers consist of immature embryos, which undergo synchronous maturation in the presence of ABA and elevated osmoticum (Attree and Fowke, 1993). The mechanism by which ABA is involved in embryo maturation is probably by preventing cleavage of the embryos giving rise to precocious maturation, and the consequent development of individual somatic embryos (Boulay *et al.*, 1988).

7.1 Materials and Methods

Sterilisation of Plant Material

The seed coats were removed and the gametophyte and embryo sterilised as in Appendix 6.1. In some experiments, the sterilised tissues were soaked overnight in sterile distilled water. The seeds used in Experiment 14 were subjected to a humidifying pre-treatment. The seed was humidified for 24 hours in a desiccator containing water as follows: a wire gauze was fitted inside the desiccator about 5 cm above the water level. The seed was placed on the gauze and the desiccator was closed tightly so that the high humidity inside the desiccator was maintained. The seeds were then sterilised as above without an overnight soak. After sterilisation, the gametophyte was split, the embryo was removed and transferred to the incubation medium. All media were prepared from the commercially available mixtures (Sigma Chemical Company).

The various experiments performed and the techniques used are summarised in Table 7.1.

Table 7.1 Summary of techniques and experimental conditions

Expt. No.	Media	Media Preparation	Growth Regulators	Seed Lot	Pretreatments	Temp °C	Light/ Dark	No. of culture
1	MS	MS basal medium containing 3% sucrose and 1% agar at pH 5.5 as in Appendix 6.2	BA = 20 µM KIN = 20 µM 2,4-D = 50 µM	A1	Overnight soak and as in Appendix 6.1	20	Light = 20 Dark = 20	40
2	MS	MS basal medium containing 3% sucrose and 1% agar at pH 5.5 as in Appendix 6.2	BA = 20 µM KIN = 20 µM 2,4-D = 50 µM	A1	Overnight soak and as in Appendix 6.1	20	Dark	60
3	MS	MS basal medium containing 3% sucrose and 1% agar at pH 5.5 as in Appendix 6.2	BA = 20 µM KIN = 20 µM 2,4-D = 50 µM	A1	As in Appendix 6.1 but not soaked	28	Dark	25
4	MS	MS basal medium containing 3% sucrose and 1% agar at pH 5.5 as in Appendix 6.2	BA = 20 µM KIN = 20 µM 2,4-D = 50 µM	A2	As in Appendix 6.1 but not soaked overnight	28	Dark	25
5	MS	MS basal medium containing 3% sucrose and 1% agar at pH 5.5 as in Appendix 6.2	BA = 20 µM KIN = 20 µM 2,4-D = 50 µM	B1	As in Appendix 6.1 but not soaked overnight	28	Dark	25
6	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 2.22 µM 2,4-D = 9.05 µM	A2	As in Appendix 6.1 but not soaked overnight	28	Dark	50
7	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 2.22 µM 2,4-D = 9.05 µM	A2	As in Appendix 6.1 soaked overnight	30	Dark	100

Table 7.1 (continued)

8	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 2.22 µM 2,4-D = 9.05 µM	A2	As in Appendix 6.1 but not soaked overnight	30	Dark	120
9	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 2.22 µM 2,4-D = 9.05 µM	A2	Overnight soak and as in Appendix 6.1	30	Dark	80
10	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 20 µM 2,4-D = 50 µM	A2	As in Appendix 6.1 but not soaked overnight	30	Dark	50
11	MS	MS basal medium containing 3% sucrose, 1% agar and 1.46 mg/ℓ filter sterilised glutamine at pH 5.5 as in Appendix 7.1	BA = 20 µM 2,4-D = 50 µM	A2	As in Appendix 6.1 but not soaked overnight	30	Dark	42
12a1	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A2	Soaked overnight	30	Dark	20
12a2	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Not soaked	30	Dark	10
12a3	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Soaked overnight	30	Dark	30
12b1	MSG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A2	Soaked overnight	30	Dark	20
12b2	MSG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Not soaked	30	Dark	10

Table 7.1 (continued)

12b3	MSG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Soaked overnight	30	Dark	30
12c1	MS	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A2	Soaked overnight	30	Dark	20
12c2	MS	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Not soaked	30	Dark	10
12c3	MS	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Soaked overnight	30	Dark	30
12d1	SH	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A2	Soaked overnight	30	Dark	20
12d2	SH	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Not soaked	30	Dark	10
12d3	SH	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Soaked overnight	30	Dark	30
12e1	LM	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A2	Soaked overnight	30	Dark	20
12e2	LM	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Not soaked	30	Dark	10
12e3	LM	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A3	Soaked overnight	30	Dark	30
13a1	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	A4	Not soaked	30	Dark	10
13a2	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 20 µM	A4	Not soaked	30	Dark	23

Table 7.1 (continued)

13a3	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 30 µM	A4	Not soaked	30	Dark	23
13a4	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 40 µM	A4	Not soaked	30	Dark	23
13b1	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 10 µM	B2	Not soaked	30	Dark	9
13b2	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 20 µM	B2	Not soaked	30	Dark	25
13b3	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 30 µM	B2	Not soaked	30	Dark	25
13b4	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 40 µM	B2	Not soaked	30	Dark	25
14a	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 20 µM	A3	Humidified at 82% RH for 24 hours	26	Dark	15
14b	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 30 µM	A3	Humidified at 82% RH for 24 hours	26	Dark	15
14c	WPMG	As in Appendix 7.1	BA = 5 µM 2,4-D = 40 µM	A3	Humidified at 82% RH for 24 hours	26	Dark	15

7.2 Results

The results of the experiments done are summarised in Table 7.2. The detailed results of each experiment are presented below.

Experiment 1

Twenty embryos were incubated in the light and 20 have been in the dark at 20 °C. Most of the embryos both in the dark and light conditions were swollen, especially in the hypocotyl region. Some embryos appeared to have developed new tissues, especially from the suspensor region. The new tissues turned brown in the long run without much increase in mass. Two embryos from the light condition lost identity but without further growth and development. An embryo was said to have lost its identity when growth made the cotyledons, hypocotyl and radicle no longer recognisable. None of the cultures was contaminated. The experiment was assessed for 23 weeks. There was no development of embryogenic tissues.

Experiment 2

Most of the embryos were swollen particularly in the hypocotyl region. It appeared that the new cells and tissues developed from the cotyledon, hypocotyl, radicle and suspensor areas but no further development occurred. A few cultures turned brown-yellow while some others showed browning. None of the cultures lost identity and three were contaminated. The cultures were kept for 18 weeks for assessment. None of the cultures responded with any kind of callus or embryogenic tissue development.

Experiment 3

A few embryos were swollen, mainly in the hypocotyl regions. New tissues appeared to have developed from the suspensor, hypocotyl and cotyledon regions in many of the embryos. The radicles of a few embryos showed new tissues developing. None of the embryos lost its identity and one was contaminated. No viable callus growth was seen in the culture. The cultures were incubated for 19 weeks for

observation. Some embryos turned brown, particularly after week 8. There was no embryogenic development.

Experiment 4

Many embryos were partly damaged when they were excised. Few cultures showed hypocotyl swelling. In some cultures new tissues developed, especially from the suspensor, radicles and hypocotyls. Some cultures turned brown and no further development took place. None of the changes was due to callus formation. The cultures were kept for 19 weeks and none of the embryos lost its identity. There was no contamination of the cultures in this experiment. There was no development of any kind of embryogenic tissues.

Experiment 5

Two embryos were contaminated. Very few embryos appeared to show new tissue formation. There was some damage on some parts of some embryos which occurred during extraction from the gametophyte. Some cultures showed browning at the cotyledon tips. Some others were yellowing. In some cultures, swelling occurred in the hypocotyl region. There was no development of embryogenic tissues.

Experiment 6

The medium used in this experiment differed from those used in the previous experiments in that it contained glutamine. Some cultures were swollen and 11 of them developed callus. Callus developed from all parts of the embryo except the suspensor. The zygotic embryos were swollen and lost identity in three weeks in about 50% of those which formed calluses. Most of the calluses subsequently turned brown. About 22% of the cultures were callused after six weeks. The cultures were subcultured in week 6 but there was little growth and development after subculturing. The cultures showed no further development 13 weeks after subculturing and most cultures degenerated after 19 weeks. There was no embryogenic development.

Expt. No.	Seed Lot	Pre-soaked	Media/Growth Regulators	Response/callus %
1	A1	Soaked	MS/BA, KIN, 2,4-D	0
2	A1	Soaked	MS/BA, KIN, 2,4-D	0
3	A1	No	MS/BA, KIN, 2,4-D	0
4	A2	No	MS/BA, KIN, 2,4-D	0
5	B1	No	MS/BA, KIN, 2,4-D	0
6	A2	No	MS + Glutamine/BA, 2,4-D	22
7	A2	No	MS + Glutamine/BA, 2,4-D	42
8	A2	No	MS + Glutamine/BA, 2,4-D	41
9	A2	No	MS + Glutamine/BA, 2,4-D	20
10	A2	No	MS + Glutamine/BA, 2,4-D	2
11	A2	No	MS + Glutamine/BA, 2,4-D	10
12a1	A2	Soaked	WPMG/BA, 2,4-D	50
12a2	A3	No	WPMG/BA, 2,4-D	40
12a3	A3	Soaked	WPMG/BA, 2,4-D	87
12b1	A2	Soaked	MSG/BA, 2,4-D	0
12b2	A3	No	MSG/BA, 2,4-D	20
12b3	A3	Soaked	MSG/BA, 2,4-D	23
12c1	A2	Soaked	MS/BA, 2,4-D	5
12c2	A3	No	MS/BA, 2,4-D	10
12c3	A3	Soaked	MS/BA, 2,4-D	43
12d1	A2	Soaked	SH/BA, 2,4-D	5
12d2	A3	No	SH/BA, 2,4-D	30
12d3	A3	Soaked	SH/BA, 2,4-D	40
12e1	A2	Soaked	LM/BA, 2,4-D	0
12e2	A3	No	LM/BA, 2,4-D	0
12e3	A3	Soaked	LM/BA, 2,4-D	3
13a1	A4	No	WPMG/BA; 2,4-D = 10 µM	10
13a2	A4	No	WPMG/BA; 2,4-D = 20 µM	4
13a3	A4	No	WPMG/BA; 2,4-D = 30 µM	26
13a4	A4	No	WPMG/BA; 2,4-D = 40 µM	13
13b1	B2	No	WPMG/BA; 2,4-D = 10 µM	44
13b2	B2	No	WPMG/BA; 2,4-D = 20 µM	24
13b3	B2	No	WPMG/BA; 2,4-D = 30 µM	24
13b4	B2	No	WPMG/BA; 2,4-D = 40 µM	16
14a	A3	No	WPMG/BA; 2,4-D = 20 µM	67
14b	A3	No	WPMG/BA; 2,4-D = 30 µM	40
14c	A3	No	WPMG/BA; 2,4-D = 40 µM	47

Table 7.2 The response of zygotic embryos to plant growth regulator treatment in the various experiments performed.

Experiment 7

In this experiment, the seed was soaked overnight in distilled water before extraction of the zygotic embryos from the gametophyte. New tissues developed from radicle, cotyledon and hypocotyl in most cultures. The overall response was that 42% of the cultures developed callus. Twenty-seven out of 42 calluses browned. Seven cultures developed sugar-like calluses and three cultures showed splitting of the epidermis. The remaining callused cultures changed slowly. In the first week, most of the changes started in the radicle which bent followed by a yellowing in colour. In some cultures the bending started from the hypocotyl. Most of the developing cultures lost identity after two weeks and all after three weeks. Some calluses grew faster compared to others. Some cultures had elongated cells, but they did not show any sign of embryogenic tissues. Only two cultures were contaminated. The explants were maintained in culture for 13 weeks.

Experiment 8

In the first week, about 20% of the cultures had either hypocotyl swelling or callus formation. About 41% of the cultures formed callus by the end of the fourth week. Various developmental forms happened after week 3. A few cultures developed sugary callus and a few of the cultures had a split epidermis. The majority of the calluses grew well and the colour of the cultures was either beige or brown. The beige coloured cultures grew faster compared to the brown ones. Some cultures developed elongated tissues after week 4. A few cultures continued development after week 5 while the majority of them showed no noticeable change and many of them turned brown. By the end of week 8, about 9% of the embryos appeared to have developed organised growth. The remaining cultures had shown no further change. The cultures were assessed at the end of week 12 and about 10% of the cultures developed organised structures on the calluses. It was thought that they were probably organogenic and were transferred to hormone free medium with activated charcoal and incubated at 20 °C in the light. Assessments were done every week for growth and development. A few cultures developed differentiating tissues. Most of the cultures turned green but the tissues did not develop into shoots as might

be expected when they were transferred to hormone free medium. The cultures showed no further change and some cultures appeared to degenerate after 3 weeks after transferring to hormone free medium. The explants were in culture for 15 weeks. There was no embryogenic development.

Experiment 9

About 20% of the cultures developed callus, mainly initiated from the radicle region of the zygotic embryo by the end of week 2. All callused zygotic embryos lost identity by week 3. A few cultures continued development while some others did not show good development of the tissues. After week 6, about 15% of the cultures appeared to have formed organised structures on the callus, but there was no clear differentiation of the tissues. After week 7, the cultures were transferred from the incubation temperature of 30 °C in the dark to a growth room at 20 °C in the light without change of the media. Close observation was made for the formation of any embryogenic tissues. After the transfer from the dark to the light, some cultures appeared to have developed elongated organised structures on the surface of the calluses. However, there was no further growth and development into any recognisable structure, for up to 10 weeks in culture.

Experiment 10

The embryos were extracted from the gametophyte soon after surface sterilisation. Two percent of the zygotic embryos at least developed calluses. The other zygotic embryos did not show any kind of change and maintained identity when they were assessed subsequently every week for 5 weeks. Five cultures were contaminated. The cultures were discarded after the end of week 5 as they did not show any development.

Experiment 11

The seed was sterilised twice (before and after overnight soaking) before extraction from the gametophyte. Ten percent of the zygotic embryos formed beige

callus after about 3 weeks. The subsequent development was slow and many of the calluses had turned brown after 6 weeks. There was no embryogenic development.

Experiment 12

This experiment was designed to test the effect of a variety of media on the induction of somatic embryogenesis on zygotic embryos extracted from two seed lots, A2 and A3. Seed lot A2 was soaked overnight before the zygotic embryo was extracted whereas seed lot A3 was subjected to an overnight or no soak. The media used were Woody Plant Medium with filter sterilised glutamine, Murashige and Skoog Medium with and without filter sterilised glutamine, Schenk and Hilderbrandt Medium and Litvay's Medium. All were supplemented with 5 μM BA and 10 μM 2,4-D. Cultures that did not form any embryogenic development after 12 weeks were discarded.

(a) Woody Plant Medium and Glutamine with filter sterilised glutamine (WPM)

1. *A2 soaked seed* - Twenty seeds were used for the experiment. Fifty percent of the embryos treated were callused (Table 7.2). The cultures did not show good growth and development and turned brown and subsequent growth appeared to be inhibited.
2. *A3 unsoaked seed* - Only 10 seeds were used in the experiment. The response in terms of callus formation was 40%. However, these cultures did not form embryogenic development.
3. *A3 soaked seed* - Thirty seeds were used for this experiment. The responses of the zygotic embryos to this medium was that 87% callused. Those cultures which showed good growth and development were subcultured. Five cultures from this experiment, numbers 5, 9, 14, 20 and 22 showed good growth and were subcultured three times into the same concentration of 2,4-D (10 μM) and BA (5 μM). Subsequently, they were subcultured onto 20 μM 2,4-D and 5 μM BA and incubated at 20 °C in the light. The five cultures that developed well were selected and

become clones 1-5 of the subsequent clone bank (Table 7.3). The remaining cultures did not develop embryogenic tissues.

(b) Murashige and Skoog and filter sterilised Glutamine (MSG)

1. *A2 soaked seed* - Twenty seeds were used for the experiment. None of the zygotic embryos showed any kind of response.
2. *A3 unsoaked seed* - Ten seeds were used for the experiment. The response was that 20% of the embryos were callused without further development. Many of them subsequently turned yellow and brown. They did not form any embryogenic tissues.
3. *A3 soaked seed* - Thirty seeds were used for the experiment and 23% of the embryos were callused. A few embryos grew white sugar-like callus and browning was common in many cultures. There was no embryogenic development.

(c) Murashige and Skoog (MS)

1. *A2 soaked seed* - Twenty embryos were used in this experiment and 5% of the embryos formed callus. There was no further development of the cultures. All calluses turned brown and no noticeable change was observed during the incubation period.
2. *A3 unsoaked seed* - Ten seeds were used for this experiment and the response was that only 10% formed a callus. The embryo which was callused in this experiment turned brown without further development and differentiation.
3. *A3 soaked seed* - Thirty seeds were used for the experiment. Forty-three percent of the zygotic embryos callused. Growth and differentiation was very poor and browning of the callus was common. A few embryos showed epidermal splitting. Most calluses appeared to degenerate after week 8. There was no embryogenic development.

(d) Schenk and Hilderbrandt (SH)

1. *A2 soaked seed* - Twenty embryos were tested for embryogenesis. The result was that only 5% of the embryos developed callus but the callus did not develop further. The callus turned brown gradually, subsequent growth was reduced and there was no development of embryogenic tissues.
2. *A3 unsoaked seed* - Ten embryos were used in this experiment and three formed calluses. One of the cultures turned brown and not much further development was observed. The second embryo turned light green in the cotyledon region and epidermal split at the radicle end. The third embryo did not show any further development. There was no development.
3. *A3 soaked seed* - Thirty seeds were used and 40% of the embryos showed at least callus formation. Many of the zygotic embryos produced brown callus but no further development was observed. A few cultures developed light-green tissues after week 5. Two cultures showed splitting of the epidermis. One of the cultures developed brown friable callus. Most of the cultures turned dark brown after week 6 and no visible change was observed. There was no embryogenic development.

(e) Litvay Medium (LM)

1. *A2 soaked seed* - Twenty seeds were used for the experiment. None of the cultures showed any sort of response to the medium.
2. *A3 unsoaked seed* - Ten zygotic embryos were tested and none of the cultures showed any response.
3. *A3 soaked seed* - Thirty zygotic embryos were tested and one formed callus by week 4. The callus turned dark brown and there was no further development.

Experiment 13

This experiment was designed to test the effect of varying concentrations of 2,4-D and 5 μM BA in WPM with filter sterilised glutamine on seed lots A4 and B2.

The seed was not previously stored. Cultures that did not show any embryogenic development were discarded after 12 weeks. Zygotic embryos that showed good development were subsequently subcultured onto WPMG containing 20 µM 2,4-D and 5 µM BA and incubated in the light at 20 °C.

a1) A4 seed on 2,4-D (10 µM)

Ten zygotic embryos were treated and 1 callused. The culture that was callused turned brown. The remaining embryos showed no visible change. There was no embryogenic development.

a2) A4 seed on 2,4-D (20 µM)

Twenty-three zygotic embryos were treated but only one produced callus. There was no further growth and differentiation of the culture and it turned brown. There was no development of embryogenic tissues.

a3) A4 seed on 2,4-D (30 µM)

Twenty-three zygotic embryos were treated and 26% formed callus. Most of the calluses developed from the radicle-hypocotyl region and browning was common, usually after week 6. The cultures did not develop further to form embryogenic tissues.

a4) A4 seed on 2,4-D (40 µM)

Out of the 23 embryos inoculated onto the medium, 13% showed some sort of callus formation. Some calluses showed epidermal splitting in the region of radicle-hypocotyl areas. Only one culture developed some green structures. The cultures did not develop further to form embryogenic tissues.

b1) B2 seed on 2,4-D (10 µM)

The number of seeds used in this experiment was 9. The response was that 44% of the cultures callused. These cultures grew quickly and lost identity by

week 6. The rates of development of these cultures were different. One culture developed greening cotyledons and shoot-like structures. The second developed brown callus and increased in size. The third developed grey-green callus and grew fastest of all. The fourth developed light-brown callus. These cultures were added to the clone bank (Table 7.3).

b2) B2 seed on 2,4-D (20 μM)

Twenty-five zygotic embryos were treated and produced some kind of callus. Some cultures grew relatively well, but none of these cultures produced any organised structure. There was no embryogenic development.

b3) B2 seed on 2,4-D (30 μM)

Twenty-five zygotic embryos were treated and 24% produced callus. There was no subsequent growth of the callus and browning developed. Some cultures had just a swollen hypocotyl for up to six weeks and showed very little change. There was no embryogenic development.

b4) B2 seed on 2,4-D (40 μM)

Twenty-five zygotic embryos were treated and 16% formed callus. Yellowing occurred at an early stage of development, while browning developed after a few weeks. There was no embryogenic development.

Experiment 14

The experiment was designed to test the effect of varying concentration of 2,4-D and 5 μM BA in WPM with filter sterilised glutamine on seed lot A3. The seed was taken from the storage at 10 °C after six and a half months. The seed was humidified prior to extraction of the embryo from the gametophyte. The cultures were incubated at 20 °C in the light. Those cultures which showed good growth and development were subcultured onto 20 μM 2,4-D plus 5 μM BA. These cultures

were added to the clone bank (Table 7.3). The cultures which did not show any development were discarded after 8 weeks.

a) 2,4-D (20 μM)

Out of 15 zygotic embryos treated, 87% of them formed calluses. Ten cultures showed further growth and development. The remaining cultures turned brown and subsequently stopped growth. Others did not show any change at all.

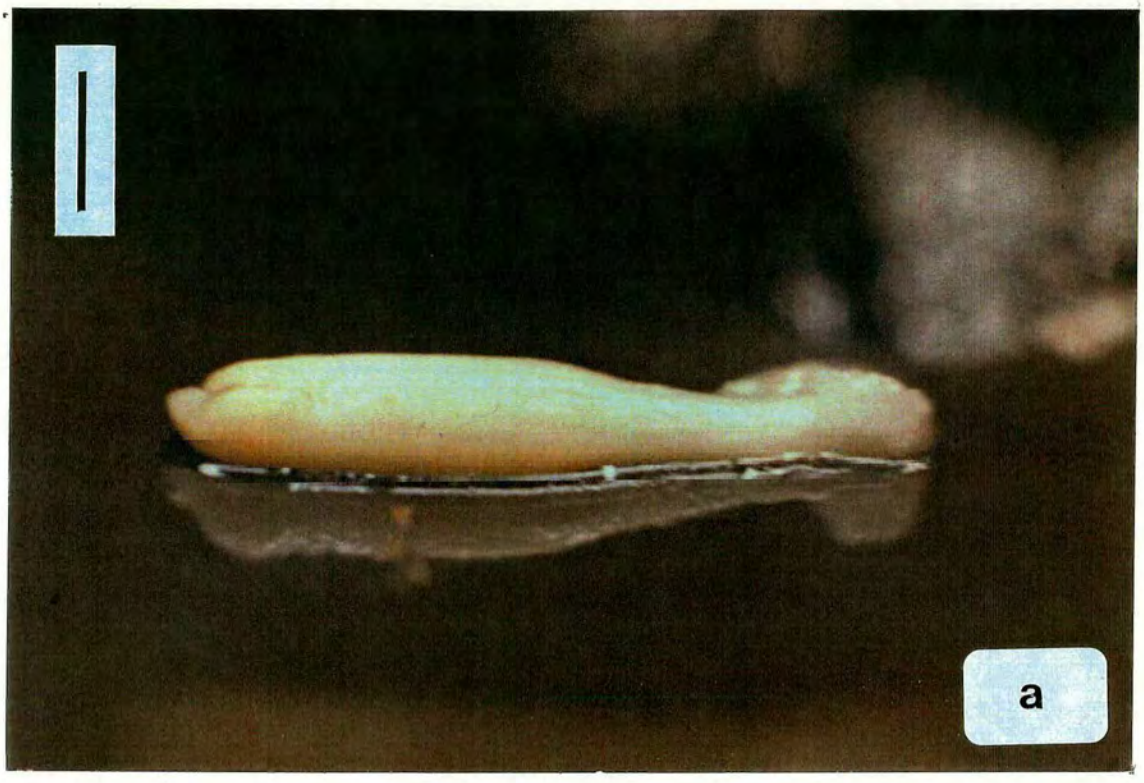
b) 2,4-D (30 μM)

Fifteen zygotic embryos were treated and 40% formed calluses and developed relatively well. The remaining cultures showed no change.

c) 2,4-D (40 μM)

Fifteen zygotic embryos were treated and 47% formed calluses. Some of the growing seven cultures showed beige colour while others showed beige-brown colour. The remaining eight cultures did not show any change.

The cultures which showed good growth from Experiments 12, 13 and 14 were subcultured several times. Some of these cultures developed embryo-like structures. Clone 8 (Table 7.3) proliferated somatic embryos from the callus. These embryos developed cotyledon and hypocotyl parts but no visible roots. Some cultures of this clone were subcultured onto hormone free medium and incubated at 20 °C both in the light and dark. Those in the dark did not show any development and gradually degenerated. The ones in the light tended to show shoot growth and development, one of which developed an elongated shoot. Figure 7.1 shows the somatic embryos at different stages of development. The zygotic embryo is slender with a splitting cotyledon and a tapering end of the radicle (Figure 7.1a). However, the somatic embryo tapers at the cotyledon end (Figure 7.1d) and this is possibly due to an incorrect auxin/cytokinin ratio or a high concentration of 2,4-D (L.H. Mo, Swedish Univ. Agric. Sci., Dept of Forest Genetics, pers. comm., 1995).


a


b

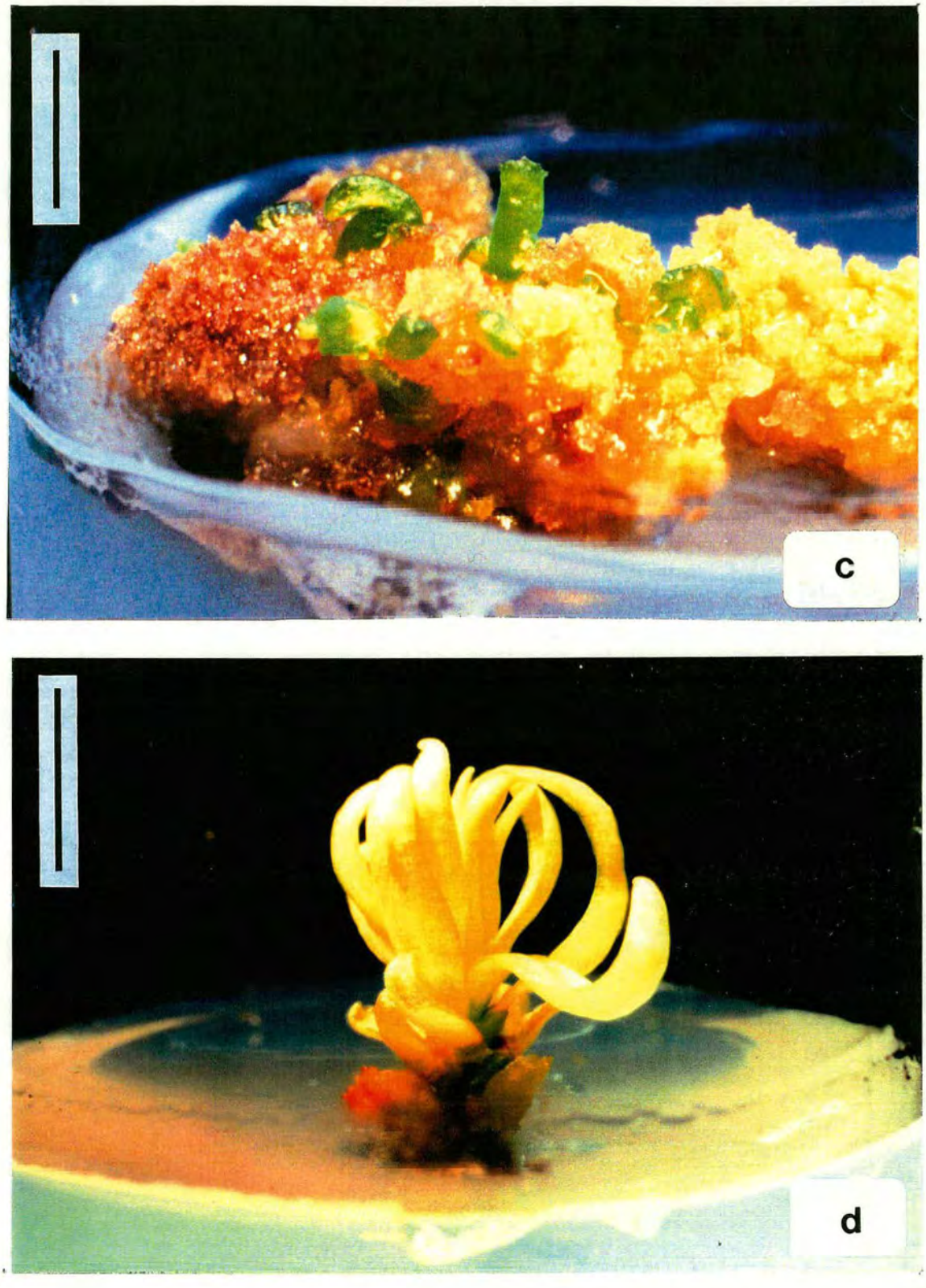


Figure 7.1 (a) Zygotic embryo (bar: 1 mm), (b) Proembryos (bar: 5 mm), (c) Green cotyledons (bar: 3mm), (d) Somatic embryos (bar: 5 mm). The cultures in a, b and c were incubated in WPMG containing 2,4-D and BA, whereas the somatic embryo in d was on MS medium without growth substances.

Clone Table

Clone No.	Culture No.	Experiment No.	Seed Lot	2,4-D Conc. µM	Medium
1	5	12	A3		
2	9	12	A3		
3	14	12	A3	10	WPMG
4	20	12	A3		
5	22	12	A3		
6	2	13	B2		
7	3	13	B2	10	WPMG
8	7	13	B2		
9	9	13	B2		
10	4	14	A3		
11	5	14	A3		
12	7	14	A3		
13	8	14	A3		
14	9	14	A3	20	WPMG
15	10	14	A3		
16	11	14	A3		
17	13	14	A3		
18	14	14	A3		
19	15	14	A3		
20	2	14	A3		
21	4	14	A3		
22	7	14	A3	30	WPMG
23	9	14	A3		
24	10	14	A3		
25	13	14	A3		
26	1	14	A3		
27	4	14	A3		
28	5	14	A3		
29	6	14	A3	40	WPMG
30	7	14	A3		
31	12	14	A3		
32	13	14	A3		

Table 7.3 Subcultured clones and their origin in experiment on embryogenesis.

7.3 Discussion

The zygotic embryos treated from three seed lots in Experiments 1 to 5 showed little response (Table 7.2). There was no loss of identity of the explants cultured for 18-23 weeks. In some cases hypocotyl swelling was observed without further expansion in size. New tissues appeared to have developed in the suspensor area which in some cases turned brown.

Experiments 6 to 11 were done on seed lot A2, which was shown to be viable using germination tests. The first 5 experiments were repeated in the next series of experiments (6 to 11) but this time with the addition of filter sterilised glutamine. It can be seen (Table 7.2) that from 2 to 42% of the zygotic embryos developed callus. It is probable that the response could be attributed to the inclusion of glutamine into the media in the second group of experiments. It has been demonstrated that most of the embryogenic cultures of loblolly pine (*Pinus taeda*) were established on the MS medium supplemented with BA and 2,4-D in the presence of filter sterilised glutamine (Becwar *et al.*, 1990).

Although they formed callus, Experiments 6 to 11 did not develop embryogenic calluses. Most of the calluses developed after swelling of the whole zygotic embryo, generally from the hypocotyl region. The colour of the embryo started to turn light yellow before the loss of identity. Most of the cultures subsequently turned brown and stopped any further expansion.

The hypocotyl regions of the zygotic embryos showed a lot of changes and in some cases seem to have undergone epidermal split. This might be a sign for early embryo formation although it was not clear from the culture. Epidermal splitting is the first sign of proembryo formation followed by the appearance of procambial bundles and the laticiferous systems, differentiation of cortical tissue, and the formation of cotyledons in *Hevea brasiliensis* (Michaux-Ferriere *et al.*, 1992).

The results of Experiment 8 confirm the above idea in that 9% of the cultures produced cotyledons (organogenic structures). These cultures could not grow further although they were transferred to hormone free medium in the presence of activated charcoal. It appears that the cultures had a maturation problem and did not respond

well when they were transferred to hormone free medium. In this group of experiments there was no clear indication for the formation of somatic embryos other than calluses and some green cotyledon-like structures.

There are several factors or combinations of them which could influence embryogenesis. Experiments 12, 13 and 14, for example, were considered with several factors, such as seed lot, media type, pre-soaking of seeds prior to embryo extraction and incubation temperature. Various levels of the growth regulator, 2,4-D were also considered. In this group of experiments, in which several combinations of media and hormone levels were used, there was some success in the initiation of somatic embryogenesis. However, subsequent maturation was a problem.

Seed lots with a high germination percentage have been shown to have the highest induction frequency of somatic embryogenesis (Tremblay, 1990; Ekberg *et al.*, 1993). However, the results obtained from the seed batches A2 and A3 did not conform to the above reports. The seed batch A3 was tested and had a low germination rate, but showed the highest response to embryogenic induction compared to A2 which was tested for a high germination rate. Embryogenic induction is at the cellular level and it could be possible that the seed batches with low germination percentage showed a higher response to treatment. Somatic embryogenesis is an induced regeneration from *in vitro* tissue culture, occurring either indirectly from callus, suspension, or protoplast culture, or directly from cells of an organised structure such as stem segment or zygotic embryo (Williams and Maheswaran, 1986).

Parental trees could make a difference between two seed batches as it could be possible that most seed was collected from a very limited number of trees. It has been reported that when the standard method for the production of plants via somatic embryogenesis is used, differences in the response of parent trees will contribute more or less to the yield of somatic plants (Ekberg *et al.*, 1993).

The explant type is another important factor which could determine the induction frequency in conifers. Many researchers report that the success for embryogenic induction with regard to explant depends on physiological states of the explant. Mo and von Arnold (1991) reported that one of the major limitations is that,

so far, embryogenic cultures of conifers can only be obtained from juvenile donor tissue. The most successful initiation of embryogenic callus was with immature zygotic embryos (von Arnold and Hakman, 1988; Finer *et al.*, 1989; Verhagen and Wann, 1989). If the earliest ontogenic stage of zygotic embryos gives the highest induction frequency, it is logical to consider the female gametophyte itself as an explant. The highest percentage of initiation of embryogenic callus was obtained from the cones with the mature (but still young) gametophytes that contained mostly precotyledonary zygotic embryos (Finer *et al.*, 1989). According to Becwar *et al.* (1990), the megagametophyte may supply the plant growth regulators necessary for initiation of the zygotic embryos. It is impossible to say in the seed batches used in this series of experiments what was the actual developmental stage of the zygotic embryo. The complete formation of the fruit of *A. gracilior* can take up to one year and it is uncertain in the seed batches used at what stage in this process the fruit was picked. Future work would focus on zygotic embryos from various stages of development for initiation of somatic embryos.

Pre-soaking of the seed lot A3 on WPMG and MS media (Table 7.2, Experiment 12) resulted in a significant increase in response compared with the unsoaked A3 seeds on the same media. It has been found that imbibition of seeds appears to play an important role in the induction of embryogenesis using mature embryos. Full imbibition of the seeds in liquid culture medium inhibited induction compared with imbibition in water (Tremblay, 1990).

Afrocarpus gracilior differs in response in the formation of calluses or embryogenic callus cultures to different media. In the investigation of suitable media, five of them were tested of which WPMG was proved to be the best (Table 7.2, Experiment 12). In the experiment with the combination of seed lot, soaked/unsoaked and media type supplemented with 2,4-D, it was found that seed lot A3 soaked overnight and established on WPMG with 5 µM BA plus 10 µM 2,4-D showed the highest callus induction rate of 87% (Table 7.2). It has been found that differences in media can affect the embryogenic potential of callus from *Hevea brasiliensis* (Montoro *et al.*, 1993). They reported that a friable callus indicated a high embryogenic potential which could be improved by modifying the culture

conditions i.e. when auxin or cytokinin concentrations were reduced and when high sucrose or calcium concentrations were maintained throughout the various subcultures.

Plant growth regulators play an important role in initiating callus and subsequently triggering somatic embryogenesis. Somatic embryos can sometimes be formed without going through the callus stages in certain conditions, depending on the species (Wann, 1989). The growth regulators mainly used in the initiation of somatic embryogenesis are BA and 2,4-D. It was found in the experiments on *A. gracilior* that the optimum 2,4-D concentration for callus formation varied with seed lot. 30 µM 2,4-D was the best with seed lot A4 whereas 10 µM 2,4-D with lot B2 and 20 µM 2,4-D with lot A3 were the best in those cases. It was decided to standardise the concentrations used for all subsequent subcultures and 20 µM 2,4-D was chosen. It is probable that the optimum auxin/cytokinin ratio was not found for *A. gracilior* in that only one embryogenic cell line was finally formed. Some reports indicate that when the optimum ratio of auxin/cytokinin is not achieved, it results in poor activity of the cultures. Changing the auxin/cytokinin optimum ratio for *Hevea brasiliensis* resulted in a loss of embryogenic growth and the formation of parenchymatous cells containing oxidised polyphenols (Montoro *et al.*, 1993). Similarly, callus browning in leaf explants of *Coffea arabica* was attributed to the presence of quinones, a product of the oxidation of phenols (Söndahl and Sharp, 1977). The concentrations of 2,4-D used in the experiments with *A. gracilior* were relatively high whereas some researchers have used very low concentrations of 2,4-D for initiation of somatic embryos. The highest somatic embryo production from explants and calluses of *Juglans nigra* was on media containing concentration of 2,4-D as low as 0.1 or 1.0 µM (Neuman *et al.*, 1993).

Embryogenic cultures selected from Experiments 12, 13 and 14 were subcultured with subdivision several times. Clone 8 grew much faster than the other clones and was soon established as an embryogenic cell line. It was found that the other clones degenerated on subculture and were soon lost. All subsequent studies on maturation and germination of the somatic embryos were made on cultures of clone 8.

Embryo maturation can occur without ABA treatment but it is dramatically improved with treatment (Gupta and Grob, 1995). The embryogenic cell line that was formed in *A. gracilior* was not subjected to ABA treatment in attempts to improve maturation. Somatic embryos were transferred to hormone-free medium.

Embryo maturation was a major problem in *A. gracilior*. Proembryos were formed in many cultures on hormone containing WPMG. The transfer of these developing embryos to hormone-free medium did not result in good maturation or germination. Plant regeneration from embryogenic cultures of conifers is still a problem and the least efficient step to plant regeneration is the maturation process (Mo and von Arnold, 1991). The problem of maturation can be solved to some extent by treating with ABA at various stages of somatic embryo development. Efficient development of somatic embryos of Norway spruce and loblolly pine to subsequent stages required the transfer of embryogenic tissue to medium with ABA (von Arnold and Hakman, 1988; Becwar *et al.*, 1990). It could be possible to improve the maturation process of *A. gracilior* with ABA. It is also possible that there might be other forms of treatment that might improve maturation in *A. gracilior*. Some researchers recommend ABA in combination with osmotica in their somatic embryo maturation protocol. The combination of ABA and a non-plasmolysing osmoticum (Polyethylene glycol-4000) was an effective treatment for maturation of *Picea glauca* somatic embryos (Misra *et al.*, 1993).

The mature somatic embryos of *A. gracilior* that developed on both hormone containing WPMG and hormone-free medium had green cotyledons similar to those of zygotic embryos with a short hypocotyl region and a poorly developed root region. When these embryos were subcultured onto hormone free media and incubated both in the light and dark, those in the light maintained green colour and showed poor growth while those in the dark turned brown and gradually degenerated. It is probable that they had not reached the stage of maturation required for subsequent germination and development. It is also possible that the transferred embryos had not accumulated sufficient stored reserves. Recent studies indicate that somatic embryo development may be related to the ABA-induced accumulation of relatively large amounts of storage lipids (Becwar *et al.*, 1990).

The incubation temperature and light are factors which could influence initiation and maturation of somatic embryogenesis. Higher temperatures close to 30 °C in the dark incubator did not initiate somatic embryos and most of the calluses turned brown. Similarly, 20 °C in the dark did not initiate somatic embryos. On the other hand, incubation at 20 °C in the light initiated somatic embryos at least in a few cases. It was not possible to further test other combinations of light and temperature. Although the optimum temperature was not investigated it appears that it could be some degrees above 20 °C. A 25-26 °C temperature has generally been used in most work done on conifer somatic embryogenesis (Tremblay, 1990).

As only one cell-line of induced somatic embryos was obtained, the overall result was not satisfactory. However, the fact that there was the development of proembryos in the one cell line is encouraging. Subsequent research might realise the high potential for somatic embryogenesis in this species.

7.4 Conclusions and Recommendations

1. Explants at various developmental stages were not tested in this work and there was no information at what stage of development the seed was during collection. If the seeds already tested are assumed to be mature, future research should be designed for embryogenesis studies with immature explants of this species.
2. The optimum concentration of 2,4-D required for the induction of somatic embryogenesis in this species is inconclusive from the data obtained in the experiments reported here. It is possible that this species requires a very low concentration of 2,4-D for successful initiation of embryogenic tissue. It is equally possible that it might require a very high concentration of 2,4-D. Future research should investigate this further.
3. The incubation temperatures of 20 and 30 °C used in this experiment appear to be too low and too high respectively. It is possible that the optimum temperature could be between 22 and 26 °C. This should be investigated in future research programmes in this line.

4. Embryo maturation was a problem encountered in this work. Various maturation techniques (e.g. application of ABA) should be used in future studies.
5. WPMG was found to be the best medium for the induction of somatic embryogenesis of this species compared with other media tested. However, it is possible that the concentration of the medium was not right and hence, the effect of half-strength and full-strength WPMG should be investigated in future work.
6. In future research, other media should be investigated.

CHAPTER 8

Conclusions and Recommendations for Future Research and Development

The first section of this chapter (8.1) is a list of the main conclusions to be drawn from the experimental chapters. The second section (8.2) is an attempt to apply the new knowledge towards the formulation of a reforestation strategy.

8.1 Conclusions of the experimental chapters

The following is an attempt to present conclusions in the style of an Executive Summary, highlighting only the most important findings and commenting on the underlying mechanisms involved in the processes involved during seed viability tests and propagation.

Chapter 2

The purpose of this chapter is to investigate the main cause of low germinability in the three species.

Afrocarpus gracilior

(i) Dormancy was apparently the main cause of low germinability, being caused by the hard seed coat which prevents imbibition of water.

(ii) An inhibitor was isolated from the seed coat of this species that was shown to inhibit germination of wheat seed and to retard the growth of seedlings of *A. gracilior*.

(iii) For storage, the seeds need to be dried down to a moisture content of 6 to 11%. Following storage under these conditions, a germination percentage of 50-80% may be expected.

(iv) The recommended germination conditions are: germinate naked seeds at 1 cm depth in moist sand at 25-30 °C, and maintain cultures with good ventilation. Under these conditions germination takes from 11 to 25 days.

(v) In this species, it appears that germinability is determined by a factor or factors related to the seed coat. In ordinary operating conditions, with the seed coat intact as prevails in present Ethiopian methods, germination is often less than 30% unless it is carried out under controlled laboratory conditions.

(vi) Two additional criteria should be observed, although they have not been investigated experimentally, viz: (a) care must be taken to ensure that the collected seeds are not immature; (b) after collection, careful handling is necessary, avoiding excessive heat or soaking at this stage.

Ekebergia capensis

(i) Low germinability was apparently caused by microbial attack of the seed during storage. One possible mechanism is that the seed coat seems to be a poor protective cover, with cracks often in the fusion line. It appears that the seeds release exudates such as sugars and amino acids from the embryo to the surface of the seed coat through these cracks causing the proliferation of fungi.

(ii) The seeds need to be dried to the lowest possible moisture content without desiccation damage in order to control microbial activity. The storage temperature should be further investigated in combination with various moisture contents. The current recommended storage conditions are at 4 °C with a moisture content of 21%.

(iii) The recommended germination conditions are that the intact seeds should be buried at a depth of 1 cm in moist sand at 20-25 °C with good aeration.

As noted for *A. gracilior* above, careful handling of the seed is necessary between collection and storage.

Pygeum africanum

(i) Low germinability appears to be caused by both seed coat and embryo dormancy. Germination is improved by nicking the seed coat. The embryos

seem to be immature during the normal collection period and it is possible to speculate that they might have a high level of ABA which could cause inhibition of germination.

(ii) The optimal storage conditions have not been investigated. However at a storage moisture content of 20%, viability was retained well at 4 °C.

(iii) The recommended germination conditions are: nicked seeds are buried 1 cm deep in moist sand at 20-25 °C, with good aeration.

(iv) Between collection and storage, careful handling of the seed is necessary.

Evaluation of methodology used in Chapter 2

Removal or nicking of seed coats of *A. gracilior* or *P. africanum* respectively, are simple techniques which can significantly improve germination. They do however require an input from a trained workforce. Similarly, full control over seed collection and handling is likely to improve germination percentages and this too requires technical skill and infrastructural support.

Widespread sampling from a number of individual trees, of a range of provenances and in different years would have been useful as there may be interannual variability. 'Good' and 'bad' seed years have been reported for other species and seem to depend partly on year-to-year variation in the weather.

Even stricter standardisation of germination conditions would have facilitated interpretation of results. In Ethiopia, it might be difficult to standardise the environment for germination and common practice there is likely to vary enormously. For example, seed drying is done in the open by exposure to sun. This inevitably subjects the seeds to an unknown warming treatment and brings them to a moisture content which may vary between seed lots. It would have been helpful to carry out a survey of such field variation, and it is clearly desirable to standardise future practice more carefully. Similar comments can be made about seed collection.

Chapter 3

The main objectives of this chapter were to estimate the viability of *A. gracilior* seeds by the tetrazolium chloride test and to investigate the optimum storage condition of those seeds.

(i) The test gave higher values of viability than the corresponding germination percentages as, indeed, is expected because dormant seeds will still respond positively to the test.

(ii) The tetrazolium and germination test results were nevertheless strongly and positively correlated.

(iii) Tetrazolium testing is also useful for establishing storage longevity. In this work the tetrazolium test indicated that the optimum storage condition is between a moisture content of 6-8% and the temperature of 4-10 °C.

Chapter 4

The main aims of this chapter were to investigate the optimum storage conditions for *A. gracilior* (A2) seeds by the germination test and to explore various combinations of moisture content and temperature in order to prolong viability.

(i) The optimum storage condition was identified to be 4 °C irrespective of moisture content (6, 8, 11 and 15%).

(ii) Germination was 76% when the experiment initially started. The highest germination percentage after storage for one year was 49% following the storage condition of 6% moisture content and 10 °C.

(iii) The recommended germination conditions suggested from the results of this chapter and Chapter 5 are the same with that mentioned in Chapter 2 for this species.

Chapter 5

The purpose of this chapter was to repeat the work of Chapter 4 with a new seed lot (A3). The trend of declining viability was also studied.

(i) The optimum storage moisture content was between 6-8%, and the optimum temperature was between 4-21 °C. A moisture content of 6% appeared to be slightly damaging particularly when storage was below 4 °C. Under the optimum condition, a germination percentage of 25-62% may be expected after a storage period of one year.

(ii) The results of the three experiments (in Chapters 3, 4 and 5) are comparable to seed storage behaviour in other tropical species such as *Coffea arabica*, Ellis *et al.* (1990) and *Carica papaya*, Ellis *et al.* (1991b). *A. gracilior* shares common characteristics with these species in that they are neither 'orthodox' nor 'recalcitrant' but intermediate.

Evaluation of methodology used in Chapters 3, 4 and 5

The research carried out here has enabled the author to gain a basic knowledge of the tetrazolium chloride test and distinguish between viable and non-viable seed. As the author's experience and knowledge of the test increase, it should be possible to distinguish between poor viability due to ageing or damage by a variety of other factors.

The tetrazolium test is sufficiently easy and fast to be useful in Ethiopia, given a minimal training programme for laboratory staff.

The experiment in Chapter 4 was done on A2 seed after it had been stored for 10 months at below 10 °C. It would have been preferable if fresh seeds were available since storage prior to receiving them might have changed their characteristics.

In Chapter 4, inconsistency in germination methodology between each withdrawal affected the trend of viability (Figure 4.1). Seed viability would have been estimated with increased precision if even a rough protocol had been available prior to the experiment. Preliminary supporting experiments were not possible as seed supply was limited. The seed (A2) used in Chapter 4 showed a relatively high germination percentage. However, it is not known how widespread the sampling had been during collection, nor the method of collection and handling. Thus no recommendations on the latter points are possible.

In Chapter 5 (A3 seed was used), the optimum germination conditions determined in Chapter 4 were adopted. However, the low viability of A3 seed could be due to the use of inappropriate methods of collection and handling.

Chapter 6

The main objective of this chapter was the establishment, multiplication and rooting of *in vitro* cultured *A. gracilior*.

(i) Culture establishment from sterilised seeds was successful with less than 5% contamination.

(ii) Decapitation of the apical segment resulted in the outgrowth of one axillary shoot on the bottom segment, very often in the upper most leaf-axil.

(iii) BA initiated buds virtually at every leaf-axil, but the buds did not show any growth and development.

(iv) Spontaneous rooting of the cultures was 35-40%. However, an attempt to improve this result by application of rooting hormones was not successful.

(v) Rooting of the cultures on a 25% aqueous leachate from seed coats (A3) of the same species gave rooting of 60%. However, when this experiment was repeated on a range of leachate concentrations below and above 25% from seed coats of A2 and A3 seeds in combination, rooting percentage was less than the previous result of A3 seed alone, the highest being 43% with concentrations of 10 and 100%.

(vi) Rooting of *in vitro* propagated shoots in *ex vitro* conditions was better at 'high' light (46 µmol photons m^{-2} s^{-1}) condition compared with the 'low' light (8 µmol photons m^{-2} s^{-1}) condition. A propriety rooting powder (Seradix 2, May & Baker Ltd) appeared to be slightly inhibitory.

(vii) In the weaning of *in vitro* rooted shoots, all plants survived a gradual exposure to the natural environment. However, subsequent growth of these plants near to the heating elements in a glasshouse resulted in the death of a few plants.

Chapter 7

The aim of this experiment was to induce somatic embryos in order eventually to be able to obtain somatic seeds.

(i) Only one cell line of somatic embryos was produced although 14 different experiments were conducted.

(ii) Many proembryos were produced but did not develop into somatic embryos.

Evaluation of methodology used in Chapters 6 and 7

Culture establishment from seed to *in vitro* condition was relatively easy. The gametophyte could stand sterilisation and stirring better compared to the seedlings which were adversely affected by sterilant (0.3% $HgCl_2$) when treated for 10 minutes.

The fact that axillary shoots were formed spontaneously on the bottom segment following excision of the apical segment, suggests an especially simple technique of shoot multiplication with a high genetic stability compared to the case when growth regulators were used.

The reason why the buds initiated by BA did not show further growth and development was not established. Time did not permit a more thorough investigation into the cause of inhibition of bud outgrowth. Transfer of the cultures to a hormone-free medium did not solve the problem.

Possibly, an appropriate concentration of the rooting hormone (IBA, NAA or their combination) would have improved the rooting percentage of this species. There is no adequate explanation as to what caused the improvement of rooting when using a 25% aqueous leachate from the seed coat of A3. When leachate of A2 was taken in combination with A3, the rooting percentage was lower than for spontaneous rooting. Crude leachate could have contained both promotory and inhibitory factors, but the concentration depends perhaps on the seed lot. Although rooting *ex vitro* of this species in the 'high' light (46 μmol photons m^{-2} s^{-1}) was better, this level of light may still not be high enough to achieve high levels of rooting.

The method of weaning in the acclimatisation process appears to be good as all plants survived and resumed growth in the natural environment. Care should be taken

in the greenhouse as the plants are sensitive to heat, and, for example, heating elements should not be in close proximity to them.

Several possible factors might be involved in the explanation of why only one cell line of somatic embryos was obtained, despite the considerable effort devoted to the subject. Many proembryos were obtained but they failed to develop into somatic embryos. One of the important factors which might have promoted their development could have been the application of ABA in the presence of polyethylene glycol-4000 (as others have tried for other species) to improve maturation. Another possible factor might be hormone concentration. The correct concentrations of the hormones might have substantially influenced the result, as might have the use of the correct developmental stage of explant, incubation temperatures, media etc. Further work is clearly required in the future, in order to develop this promising line of enquiry.

8.2 Seed source and reforestation strategies

Seed availability is a prerequisite for launching any reforestation programmes through seedling production. Four possible seed sources described in this chapter are: forest stands; soil seed bank; stored seed (genebank); and somatic seeds.

8.2.1 Forest Stands

Conservation and Reforestation

With continuing loss of tropical forests, it is imperative to use all possible means to conserve and develop the forest resources. The forest stand itself can be the best source of seeds for a reforestation plan. Plant genotypes could be conserved *in-situ*, and the forest is reproduced true-to-type at a relatively low cost. With logging and the loss of forest area, however, the dwindling population would result in a narrow genetic base and, possibly, the less desirable residual individuals of the remaining logged over population providing the seed source. Individuals of the remaining forests may not all produce seeds and this situation could lead to a further scarcity of seeds. Moreover, certain individuals of the forest population could produce seeds which have genetically poor germinative power. The degradation of forest might be

accompanied by changes of microclimate and soil erosion to the point where the ecosystem cannot recover. Among the many problems of reforestation are the absence of easy propagation techniques and inadequate knowledge of seed dormancy of difficult-to-germinate species.

Interrelationships within the ecosystem should not be ignored in conservation and development of forest vegetation. The existence of wildlife in a certain forest ecosystem is not only of benefit for food and shelter but also contributes to the existence of vegetation. Some wildlife are involved in pollination (e.g. birds and insects), some in seed dispersal (e.g. primates, birds, ants, etc) and others in germination (e.g. primates, birds, etc). The loss of any one community in the ecosystem can sooner or later affect the system itself. The forest ecosystem can be maintained and developed by carefully designed management plans provided these are fully implemented and supported by legally binding regulations. The main problem with many tropical forests is the lack of commitment from the Governments to implement existing rules according to the drafted strategies, and this is true of Ethiopia's Forestry Programme.

For example, the replacement of fuelwood by, for example, solar, wind and hydro-electric power could save a considerable amount of forest vegetation from being cut. In Ethiopia, forest felling before 1974 was mainly because of the expansion of farm land and clear-felling for timber and fuelwood. The magnitude of clear-felling after 1974 has increased because of a common land policy, villagisation programme and the involvement of state farms for cultivation of food crops in addition to the previous forms of deforestation.

Today, the remaining, scanty natural forests of Ethiopia are confined mainly to the central-western plateau, further to the south-west and to the south-eastern plateau across the rift-valley. The rift-valley is predominantly covered by *Acacia* woodlands. Most of the existing forests are under pressure from one form of disturbance or another and it is imperative to design a strategy to save them as a seed source to initiate reforestation programmes. Some tree species are endangered, or even threatened with extinction, and plans should be laid down to allocate limited resources to saving the many endangered and valuable species by launching reforestation

activities. Short and long term plans are necessary to work towards seed production from the existing and recovering forests. Dormancy and germination problems of seeds should be studied in the meantime. More land should be allocated to forest vegetation to avail seed production of endangered species and it is necessary to stop clear-felling of indigenous species for timber and other purposes. Alternative supplies of timber could be plantations or, temporarily, imports. People living close to the natural forests should be given necessary assistance, like employment benefit, to ease the pressure to deforest the new land for farms. Government mass media may need to advocate the necessity of conserving the remaining forests and developing forest resources in general: private organisations and individuals might be encouraged by material and moral incentives, e.g. people may be encouraged to construct houses and fences out of slabs and bricks. It should be noted that every move towards the alternatives to the clear-felling of forests can contribute to conservation of natural vegetation *in situ*. Plans are needed to prioritise the potential forest sites for future seed production based on previous research findings (Blyth, 1991).

8.2.2 Soil seed bank

Places once dominated by forest cover can contain a large quantity of seeds buried in the soil for many years. The abundance of seed in the soil depends on the seed production potential of each species. Some species produce huge quantities of seed each year, which are adapted to wide dispersal, while others produce seeds less often or in small quantities but with the capacity to lie dormant in the forest floor for a number of years (Marquis, 1975). Seeds buried in the soil with little disturbance and low light intensity tend to go into dormancy: the effect of continuous darkness is to inhibit germination, and this may also explain how germination is initiated when disturbance exposes buried seeds at the soil surface (Grime, 1981).

Soil seed banks are an important part of the flora of a site ecosystem and the effectiveness of the seed bank contribution to regeneration should be appreciated. In a world increasingly disturbed by human activities, it is inevitable that seed banks will frequently be important in the management and restoration of vegetation (Thompson,

1992). They are part of the forest conservation strategy by which endangered species can be regenerated: catastrophic events like fire and drought can destroy vegetation but buried seeds can still remain viable in the soil. The persistent seed bank probably functions as a buffer against population extinction in precariously exploited habitats (Grime, 1981).

In a forest regeneration programme, it is not desirable to rely on the soil seed bank as a source of seed. There could be a wide difference between ages of seeds as they have accumulated over many years and it might not be appropriate to apply a single specific treatment to the seed population to break dormancy of various ages. Because of the adaptive significance of dormancy in various types of seeds, plant communities may have quite different types of seed bank. Germination in the soil may be caused by the pre-existing phytochrome (Pfr) in the seed and this can disappear by dark-reversion and dark-dormancy can be induced (Pons, 1992). Pons (1992) further indicated other forms of dormancy caused by germination inhibiting conditions such as unsuitable temperature and low water potential. Interaction of many factors is responsible for the dormancy of seeds in the soil seed banks, e.g. light, temperature, water potential, nitrates, genotypes, soil depth, oxygen, etc. Various types of soil disturbance may bring about conducive environments for breaking seed dormancy: clear-felling results in regeneration with fewer species and slow growth rate because of soil degeneration - logged areas do revegetate fairly rapidly, but the new growth is poorer and less diverse (Saulei, 1984). Where the objective of management is to create or maintain a diversity of species, it may be necessary to apply a variety of forms of disturbance (Grime, 1981): removal of debris, land scarification and many other disturbances could be applied to initiate forest regeneration.

In regenerating forests, pioneer species usually emerge faster and shade-demanding species later, provided that there are viable seeds in the seed bank and the right conditions for germination. Those species that could not be regenerated given the possible disturbance treatments can be planted into the area: sites which have been regenerated with a low species diversity could also be enriched by planting. Animals which used to inhabit the previous forest should be reintroduced to initiate

the restoration of the ecosystem and once regeneration is initiated the area should be protected from any destructive interference.

In Ethiopia, several pilot studies on regeneration have given encouraging results. Regeneration trials of *Acacia* in some places in the rift valley have been a good start and the results are found to be promising. Highland species might be regenerated probably with a less species diversity. *Afrocarpus gracilior* appears to have regenerated fairly well in the forest where the seeds were buried in the soil: the seeds seem to stay viable for several years provided that they find their way down into the soil with accumulation of thick layers of organic matter. In compact soil under the forest cover where disturbance by animals is acute, there is little regeneration of *Afrocarpus*.

Lands cultivated for many years have less chance of forest regeneration. There is a need for research in this area to estimate the population of buried seeds: data of this sort are important to help draft a management plan for forest regeneration. It is also important to know the regeneration potential of a species before launching reforestation activities. With species of high regeneration potential, reforestation can be carried out from the soil seed bank at a relatively low cost.

8.2.3 Stored seed [gene bank]: *Ex-situ* conservation

As tropical forests are declining, there is world-wide interest to keep seeds, particularly of endangered species, in suitable storage conditions (Thornhill and Koopowitz, 1992). Seed storage would appear to be a relatively easy and less expensive method of preserving plant genotypes compared with other *ex situ* conservation strategies. In Ethiopia, the activity of forest seed procurement has been undertaken by the Forestry Research Centre, while forest development activities have been carried out by the State Forest and Soil and Water Conservation Departments and some Non-Governmental Organisations. The Forestry Research Centre has been the national seed procurement and distribution body structured under the then Ministry of Natural Resources Conservation and Environmental Protection. The centre has been committed to collecting more than 50 seed species in the last two

decades, the majority of which are exotic, based on requests from tree planting bodies.

The United Nations Development Programme (UNDP)/United Nations Sudano-Sahelian Office (UNSO) understood the seriousness of forestry problems in Ethiopia and launched a 5-year project on tree seed towards the end of 1992. It is hoped that the project will lay the ground for quality seed procurement and development of working facilities. It is expected at the end of the project that seed procurement and proper management will continue not only in the Tree Seed Centre but also in the regional bases.

Forest seeds, particularly of rare species, should be preserved in a defined storage condition for each species. Many species can have the same storage condition, but this has to be proved by experimentation. The storage condition studies in this work on *A. gracilior* (and partially on *E. capensis* and *P. africanum*) should be repeated (with suitable modification) for other indigenous species of Ethiopia. It is very important in seed handling that care must be taken at every step from collection up to storage and until the seed is sown for germination. Although reforestation is recognised as an essential activity, the adequate supply of seed of high quality and high genetic potential is often a limiting factor in many countries and thus, emphasis has been placed on research project activities including production, collection, handling, processing, testing and storage of seed for a proper management (Wang, 1988). Strategies geared to such research activities should be designed for full implementation. It is better to collect a limited quantity of seed with proper handling throughout the stages of procurement than to collect a bulk quantity without a proper seed management. It is worthwhile describing these activities at every stage in seed procurement.

8.2.3.1 Seed production

Seed quality can be improved through tree improvement programmes and eventually by establishing seed orchards. Seed orchards of improved genotypes should be used as the seed source for the future plantation programme. A reasonable number of seed orchards should be established at zonal/regional levels so that future

seed collection could concentrate mainly on close proximity to the plantation areas. The existing seed production plantations can serve as transitional seed orchards or used as a base population until the proper seed orchard has materialised.

8.2.3.2 Seed collection

Seed collection should be well designed and organised. The existing practice of seed collection randomly from any site where seed is available should be improved to a system of collecting quality seed. The tradition of transporting bulk seeds a long distance to Addis Ababa needs to be improved. It might be enough to take sample seeds to Addis Ababa if need be for a laboratory test.

First of all, bulk seed which is surplus to requirements for the next planting season should not be collected (unless otherwise required). This would alleviate the storage problem and improve germination after relatively long-term storage. The fact that the proper storage conditions for many Ethiopian species are not known and the seeds are stored in unsuitable conditions probably results in more rapid deterioration than anticipated.

Precautions should be taken to follow all seed collection procedures. Only mature seeds should be collected, carefully without destroying the immature seed which should remain on the mother plant. Collection of immature seeds is not worthwhile because they cannot stand desiccation. Collection of poor quality seed results in the loss of not only time and material resources spent on collection but also one or more years of plantation programmes. It is important to use the proper seed collection equipment for the safety of the collector and for collecting seeds of the required quality.

8.2.3.3 Seed handling

After separation from the mother plants, the seeds should be handled with great care. It is always advisable to keep a small quantity of seed (particularly wet seed) in relatively small bags (e.g. 10 kg) so that air circulates in between bags in a temporary storage in the field. A large quantity of wet seed may build up heat in the middle of the bag and cause a rapid loss of viability.

Seed transportation is one of the very important procedures in seed handling. People might overlook the importance of the proper care of seed during transportation in the vehicles, ships and aeroplanes. During transportation, the seeds should be kept at an ambient condition and more importantly should not be under any pressure. The seeds are in the process of desiccation at the time of shedding and as a general rule should not be kept in wet conditions (there are some exceptions with recalcitrant seeds which should be handled by experts).

8.2.3.4 Seed processing

Methods of seed processing may differ from species to species. Some seeds are already dry at the time of shedding (many of the indigenous species of *Acacia* in Ethiopia for example) and may not need a special extraction treatment other than mechanical removal of the seeds from the pods. Many other lowland legumes like *Albizia gummifera* also have similar seed desiccation behaviour to the lowland *Acacias*. The optimum moisture content to which a seed has to be dried for storage should be experimentally investigated. The type of seed processing could also depend on the desiccation tolerance of a species.

Seed storage behaviour of many mid elevation and highland species of Ethiopia is not yet known for management decision as to how to undertake the extraction work. *Afrocarpus gracilior* seeds are semi-desiccated at the time of shedding: if seed collection is done at the time of yellowing the seeds are wetter than at the time of natural shedding. The seeds of *Afrocarpus* are traditionally soaked in water for about two days to soften the pulp for easy extraction by hand. It is not yet experimentally proven whether or not pre-soaking of *Afrocarpus* seed is the right method as far as viability is concerned. One of the side experiments in this work indicated that pre-soaking of the seed with intact pulp appeared not to have an immediate effect on seed viability: *Afrocarpus* seeds soaked for six hours after seed coat removal gave slightly higher germination percentages than the seeds soaked for 24 hours. However, the effect of pre-soaking on long-term storage remains to be seen. Experiments of this type should also be done for many other indigenous seed species as pre-soaking might be detrimental to the viability of some seeds.

The drying period accompanying pulp extraction is another thing to be investigated. Some seeds can best maintain their viability if they are dried slowly for a longer period of time before storage. Orthodox seeds appear to keep their viability when they are desiccated slowly, contrary to the recalcitrant seeds. In nature, the seeds are in a state of drying when they are shed. This process is expected to continue after shedding until the seeds come to a minimum moisture content at which viability can best be maintained. It is therefore logical to avoid pre-soaking for extraction purposes at least for those seeds which are to be stored for some time. Recalcitrant seeds are more delicate to handle: more research is needed to investigate the storage conditions for such seeds.

Appropriate seed processing facilities should be organised before any seed collection begins. Air-drying might be necessary by laying the seeds on a platform about one metre above the ground. Drying seeds on the ground under direct tropical sun light might kill the seeds due to high temperature.

8.2.3.5 Seed testing

Seed viability should always be estimated for each seed population, taking samples to carry out germination, tetrazolium chloride or other tests. As viability results are assumed applicable to the whole seed lot the tests should be done with care and efficiency, and seed testing facilities such as laboratories, nurseries and/or greenhouses should be well organised. Trained personnel are a necessity as the work requires skill and technique, in line with the standard rules of the International Seed Testing Association (ISTA). The Forest Seed Laboratory in Addis Ababa should be working to this standard.

The task of seed procurement and storage needs to be undertaken at the zonal/regional level in Ethiopia in order to reduce the risk of damage in transport. Seed testing activities may also be done at the regional level, requiring some laboratory and nursery facilities. Seed research on physiology and storage conditions are best studied in the Seed Centre at head office level, which coordinates research at the national level to categorise seeds according to their storage behaviour. Both the Centre and the regional stations should work in close collaboration: one of the tasks

of the Centre should be to offer technical and other necessary support to the regions so that some research activities which are done in the Centre might be replicated at the regional level.

8.2.3.6 Seed storage

The seeds are required to be collected based on the short and long-term storage plans. The regions would plan their annual requirement of the seed and arrange a collection programme in line with the target.

Storage facilities need to be constructed at the regional level. Stores can be constructed from local materials such as thick-walled wattle and daub roofed with grass thatches, for example. Such houses are cool enough to keep the viability of seed for at least one season until it is used to raise seedlings.

The central seed storage should be used mainly to preserve the seeds for genetic conservation. The seeds in excess of the annual requirement of either the regions or the Centre itself can be stored in the central store to be used in the next plantation season. The proper cold store and freezing facilities are used to store seeds based on priority list starting with the species threatened with extinction. Seed storage behaviour of each species needs to be worked out to avoid storing two or more seed groups with unrelated behaviour in the same conditions.

Germination and seedling physiology should also be studied to find the rapid propagation method so as to promote reforestation programmes. The task of *in situ* and *ex situ* conservation of species should be implemented side by side.

Seed storage for genetic conservation in Ethiopia has so far concentrated mainly on agricultural species. The country has a huge diversity of forest species because of the wide range of ecological zones and the seeds therefore differ in their ability to maintain their viability because of adaptation to a wide range of conditions. Many of the orthodox seeds can be stored for a long period of time by controlling the moisture content of seed and the storage temperature. In the future, liquid nitrogen could be used to store seeds for a long time. Some desiccation-tolerant species are also liquid nitrogen-tolerant and could be stored in this condition for a lengthy period of time (Wang, 1988). In future, it appears possible that recalcitrant seeds may be stored in

liquid nitrogen: embryos of recalcitrant seeds can be safely stored in liquid nitrogen if they are frozen down at a rate of about 200 °C per minute (P. Berjak, University of Natal, South Africa, personal communication, 1995). Research towards the preservation of plant materials in cold rooms, freezing chambers and liquid nitrogen are crucial for Ethiopia before it is too late to save genetic resources of rare species. At the same time, progress should be made to overcome propagation problems of indigenous species and work hard towards *in situ* conservation.

8.2.4 Somatic seeds

Plant biotechnology is progressing towards successful implementation of somatic embryogenesis techniques so as to enable a harvest of artificial seeds from somatic cells. The work on this technology should be included in the long-term strategy for plant propagation and genetic improvement. The use of micropropagation and somatic embryo cultures in reforestation strategy will be described in the next section.

8.2.5 Micropropagation and somatic embryo culture and reforestation strategy

Some of Ethiopia's indigenous tree species like *Aningeria adolfi-friederici* and *Syzygium guineense* are found among the remaining natural forests confined to very limited areas. Although there are no published reports on the storage behaviour of these species they appear to be recalcitrant, and together with other species of similar seed storage behaviour are potentially suitable for micropropagation and somatic embryogenesis. This appears to be a good option for clonal multiplication, with the potential of becoming an efficient method for plant multiplication and a successful system for studying plant development (von Arnold and Hakman, 1988). In cases where rooting and other problems become obstacles to propagation by cuttings somatic embryogenesis may be the best alternative for plant multiplication. The rapid loss of rooting potential and plagiotropic growth of many conifer cuttings severely limits the use of conventional cuttings and the recent successful induction and

regeneration of plants from embryogenic cultures of conifers has created new possibilities for clonal forestry (Klimaszewska *et al.*, 1992). Research into micropropagation and somatic embryogenesis should be planned, at least in the long-term, for *in situ* conservation of recalcitrant species in particular and very small populations.

8.2.6 Conclusions and Recommendations

Ethiopia needs preservation of plant material in the form of seed and other propagules for conservation and development programmes. This could be done in short and long-term plans:

Short-term

1. Seed collection for research and planting targets.
2. Research geared to investigation of the storage behaviour of seeds - orthodox, intermediate and recalcitrant.
3. Preservation of those seeds of desirable species whose storage behaviour is known in their optimum storage condition.
4. Establishment of seed stations at the regional level and create networks.

Long-term

1. Continued seed collection geared to quality for research and planting targets.
2. Continued research on seed storage behaviour and preserve the seeds and other plant materials in the cold store and gene bank.
3. Establishment of *in situ* conservation in parallel with gene bank storage of selected species.
4. Establishment of seed orchards to enable seed multiplication.
5. Cryopreservation and plant regeneration from micropropagation and somatic embryo cultures.

REFERENCES

Adam, A.M., Mcdonald, M.B. Jr. and Henderlog, P.R. (1989) The influence of seed position, planting and harvesting dates on soybean seed quality. *Seed Science and Technology* 17: 143-152.

Agamy, M. (1986) The effects of different soil water potential, temperature and salinity on germination of seeds of the desert shrub *Zygophyllum dumosum. Physiologia Plantarum* 67: 305-309.

Ahuja, M.R. (1993) Biotechnology and clonal forestry. In: *Clonal Forestry I, Genetics and Biotechnology* (eds. M. Abuja and W.J. Libby), pp 135-144. Springer-Verlag, Berlin.

Aiazzi, M.T. and Argiiello, J.A. (1992) Dormancy and germination studies on dispersal units of *cordobensis* (Gandoger et Sucker) (Chinopodiaceae). *Seed Science and Technology* 20: 401-407.

Akoroda, M.O. (1986) Seed desiccation and recalcitrance in *Telfairia occidentalis. Seed Science and Technology* 14: 327-332.

Arello, E.F. and Pinto, J.E.B.P. (1993) *In vitro* propagation of *Kielmeyera coriacea.* I. Effect of various concentrations and combinations of benzilaminopurine and naphthaleneacetic acid on shoot multiplication. *Pesquisa Agropecuaria Brasileira* 28(1): 25-31.

Arnold, S.V. and Eriksson, T. (1981) *In vitro* studies of adventitious shoot formation in *Pinus contorta. Canadian Journal of Botany* 59: 870-874.

Attree, S.M. and Fowke, L.C. (1993) Embryogeny of gymnosperms: advances in synthetic seed technology of conifers. *Plant Cell, Tissue and Organ Culture* 35(1): 1-35.

Auld, T.D. (1986) Dormancy and viability in *Acacia suaveolens* (Sm.) Willd. *Australian Journal of Botany* 34(4): 463-472.

Bajaj, Y.P.S. (1986) Biotechnology of tree improvement for rapid propagation and biomass energy production. In: *Biotechnology in Agriculture and Forestry I: Trees I* (ed. Y.P.S. Bajaj), pp 1-23. Springer-Verlag, Berlin.

Basu, R.N. and Pal, P. (1980) Control of rice seed deterioration by hydration-dehydration pretreatments. *Seed Sciene and Technology* 8: 151-160.

Becwar, M.R., Nagmani, R. and Wann, S.R. (1990) Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*). *Canadian Journal of Forest Research* 20: 810-817.

Becwar, M.R., Stanwood, P.C. and Roos, E.E. (1982) Dehydration effects on imbibitional leakage from desiccation-sensitive seeds. *Plant Physiology* 69: 1132-1135.

Bekele, T. (1993) Vegetation ecology of remnant afromontane forests on the central plateau of Shewa, Ethiopia. *Acta Phytogeographica Suecica* 79: 1-64.

Berjak, P., Dini, M. and Pammenter, N.W. (1984) Possible mechanisms underlying the differing dehydration responses in recalcitrant and orthodox seeds: desiccation associated sub-cellular changes in propagules of *Avicennia marina*. *Seed Science and Technology* 12: 365-384.

Berjak, P., Farrant, J.M., Mycock, D.J. and Pammenter, N.W. (1990) Recalcitrant (homoiohydrous) seeds: the enigma of their desiccation sensitivity. *Seed Science and Technology* 18: 297-310.

Bewley, J.D. (1979) Physiological aspects of desiccation tolerance. *Annual Review of Plant Physiology* 30: 195-238.

Bewley, J.D. and Black, M. (1978) *Physiology and biochemistry of seeds in relation to germination*, Vol 1. Springer-Verlag, Berlin.

Bewley, J.D. and Black, M. (1994) *Seeds: Physiology of Development and Germination*, 2nd edition. Plenum Press, New York and London.

Blyth, J.F. (1991) Forestry research consultants report, Addis-Bah Forestry Development Project, Ethiopia (April 1991). Land and Timber Services Ltd, Edinburgh.

Bonga, J.M. and Aderkas, P.V. (1992) *Forestry sciences: In vitro Culture of Trees*. Kluwer Academic Publishers, Dordrecht.

Bonner, F.T. (1986) Measurement of seed vigor for loblolly and slash pines. *Forest Science* 32(1): 170-178.

Bose,T.K. and Mandal, D.P. (1973) Rooting of cuttings under mist. *Indian Journal of Agricultural Sciences* 43(3): 229-233.

Boulay, M.P., Gupta, P.K., Krogstrup, P. and Durzan, D.J. (1988) Development of somatic embryos from cell suspension cultures of Norway spruce (*Picea abies* Karst.). *Plant Cell Reports* 7: 134-137.

Bradbeer, J.W. (1992) *Seed dormancy and germination*. Blackie Academic & Professional, Glasgow, UK.

Breitenbach, F.V. (1963) *The indigenous trees of Ethiopia*, 2nd edition. Ethiopian Forestry Association, Addis Ababa.

Bueno, M.A., Astorga, R. and Manzanera, J.A. (1992) Micropropagation of *Populus alba* 'Siberia extremena' from aments. *Investigacion Agraria, Sistemas-Y-Recurses Forestales* 1(2): 163-171.

Chalupa, V. (1987) Temperature. In: *Forestry Sciences: Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 142-151. Martinus Nijhoff, Dordrecht.

Chalupa, V. (1990) Plant regeneration by somatic embryogenesis from cultured immature embryos of oak (*Quercus robur* L.) and linden (*Tilia cordata* Mill.). *Plant Cell Reports* 9(7): 398-401.

Cheng, B., Peterson, C.M. and Mitchel, R.J. (1992) The role of sucrose, auxin and explant source on *in vitro* rooting of seedling explants of *Eucalyptus sideroxylon*. *Plant Science Limerick* 87(2): 207-214.

Chin, H.F. (1988) Storage and vigour. *Seed Science and Technology* 16: 1-4.

Chin, H.F., Hor, Y.L. and Lassin, M.B.M. (1984) Identification of recalcitrant seeds. *Seed Science and Technology* 12: 429-436.

Ching, T.M. (1966) Compositional changes of Douglas fir seeds during germination. *Plant Physiology* 41: 1313-l319.

Chou, C.H. and Waller, G.R. (1980) Possible allelopathic constituents of *Coffea arabica*. *Journal of Chemical Ecology* 6(3): 643-654.

Chowdhury, S.R. and Choudhuri, M.A. (1987) Effect of pre-soaking and dehydration on germination and early seedling growth performance of two jute species under water stress condition. *Seed Science and Technology* 15: 23-33.

Côme, D. (1983) Post harvest physiology of seeds as related to quality and germinability. In: *Post harvest Physiology and Crop Preservation* (ed. M. Lieberman), pp 165-190. Plenum Press, New York.

Corbineau, F. and Côme, D. (1988) Storage of recalcitrant seeds of four tropical species. *Seed Science and Technology* 16: 97-103.

Corral, R., Pita, J.M. and Garica, F.P. (1990) Some aspects of seed germination in four species of *Cistus* L. *Seed Science and Technology* 18: 321-325.

De Carli., M.E., Baldan, B., Mariani, P. and Rascio, N. (1987) Sub-cellular and physiological changes in *Picea excelsa* seeds during germination. *Cytobios* 50: 29-39.

Del Amo, S. and Anaya, A.L. (1978) Effect of some sesquiterpenic lactones on the growth of certain secondary tropical species. *Journal of Chemical Ecology* 4(3): 305-313.

Dickie, J.B., May, K., Morris, S.V.A. and Titley, S.E. (1991) The effect of desiccation on seed survival in *Acer platanoides* L. and *Acer pseudoplatanus* L. *Seed Science Research* 1(3): 149-162.

Dittmar, O. (1991) *In vitro* regeneration of curly birch, *Betula pendula* var. carelica. *Thaiszia* 1: 119-124.

Dodd, M.C., Staden, J.V. and Smith, M.T. (1989) Seed germination in *Podocarpus henkelii:* An ultrastructural and biochemical study. *Annals of Botany* 64: 569-579.

Dure, L.S. III. (1975) Seed formation. *Annual Review of Plant Physiology* 26: 259-278.

Edwards, C.A. and Mumford, P.M. (1985) The effect of the drying method on the behaviour of sour orange (*Citrus aurantium*) seeds. *Seed Science and Technology* 13: 227-234.

Edwards, D.G.W. (1980) Maturity and quality of tree seeds - a state-of-the-art review. *Seed Science and Technology* 8: 625-657.

Ekberg, I., Norell, L. and von Arnold, S. (1993) Are there any associations between embryogenic capacity and phenological traits in two populations of *Picea abies*? *Canadian Journal of Forest Research* 23: 731-737.

Ellis, R.H., Hong, T.D. and Roberts, E.H. (1985) *Handbook of seed technology for genebanks*. International Board for Plant Genetic Resources, Rome.

Ellis, R.H., Hong, T.D. and Roberts, E.H. (1990a) An intermediate category of seed storage behaviour. 1. Coffee. *Journal of Experimental Botany* 41(230): 1167-1174.

Ellis, R.H., Hong, T.D. and Roberts, E.H. (1991a) An intermediate category of seed storage behaviour? Effects of provenance, immaturity, and imbibition on Desiccation-Tolerance in coffee. *Journal of Experimental Botany* 42(238): 653-657.

Ellis, R.H., Hong, T.D. and Roberts, E.H. (1991b) Effect of storage temperature and moisture on the germination of papaya seeds. *Seed Science and Technology* 1: 69-72.

Ellis, R.H., Hong, T.D., Roberts, E.H. and Soetisna, U. (1991c) Seed storage behaviour in *Elaeis guineensis*. *Seed Science Research* 1: 99-104.

Ellis, R.H., Hong, T.D., Roberts, E.H. and Tao, K.L. (1990b) Low moisture content limits to relations between seed longevity and moisture. *Annals of Botany* 65: 493-504.

Ellis, R.H. and Roberts, E.H. (1981) The quantification of ageing and survival in orthodox seeds. *Seed Science and Technology* 9: 373-409.

Emmanual, C.J.S.K. and Dharmaswamy, S.S. (1991) Short note: Seed source variation in storage life of teak seeds. *Silva Genetica* 40: 249-250.

Enescu, V. (1981) Report of the forest tree seed committee working group on tetrazolium testing 1977-1980. *Seed Science and Technology* 9: 195-203.

Ezumah, B.S. (1986) Germination of storage of neem (*Azadirachta indica* A. Juss) seed. *Seed Science and Technology* 14: 593-600.

FAO (1985) *A guide to forest seed handling: with special reference to the tropics*. FAO, Rome.

FAO (1989) Update of recent development. In: *Role of Forestry in Combating Desertification, Proceedings of the FAO Expert Consultation*, pp 16-75. FAO, Rome.

FAO (1995) Forest resources assessment 1990, global synthesis. *FAO Forestry Paper 124*, pp 7-23.

Farrant, J.M., Berjak, P. and Pammenter, N.W. (1993) Studies on the development of desiccation-sensitive (recalcitrant) seeds of *Avicennia marina* (Forssk.) Vierh.: The acquisition of germinability and response to storage and dehydration. *Annals of Botany* 71: 405-410.

Farrant, J.M., Pammenter, N.W. and Berjak, P. (1986) The increasing desiccation sensitivity of recalcitrant *Avicennia marina* seeds with storage time. *Physiologia Plantarum* 67: 291-298.

Farrant, J.M., Pammenter, N.W. and Berjak, P. (1988) Recalcitrance - a current assessment. *Seed Science and Technology* 16: 155-166.

Farrant, J.M., Pammenter, N.W. and Berjak, P. (1989) Germination-associated events and the desiccation sensitivity of recalcitrant seeds - a study on three unrelated species. *Planta* 178: 189-198.

Finch-Savage, W.E. and Clay, H.A. (1994) Evidence that ethylene, light and abscisic acid interact to inhibit germination in the recalcitrant seeds of *Quercus robur* L. *Journal of Experimental Botany* 45 (278): 1295-1299.

Finer, J.J., Kriebel, H.B. and Becwar, M.R. (1989) Initiation of embryogenic callus and suspension cultures of eastern white pine (*Pinus strobus* L.). *Plant Cell Reports* 8: 203-206.

Finkelstein, R.R., Tenbarge, K.M., Shumway, J.E. and Crouch, M.L. (1985) Role of ABA in maturation of rapeseed embryos. *Plant Physiology* 78: 630-636.

Finney, D.J. (1962) *Probit Analysis*, 2nd edition. Cambridge University Press, Cambridge, 318pp.

Finney, D.J. (1971) *Probit Analysis*, 3rd edition. Cambridge University Press, London.

Finney, D.J. (1980) The estimation of parameters by least squares from unbalanced experiments. *J. Agric. Sci. Camb.* 95: 181-189.

Flandez, M.S. and Quedraogo, K. (1994) Management of woodlands and savannahs in the Sudano-Sahelian zone. In: *Reading in Sustainable Forest Management*, pp 33-43. FAO.

Fouret, Y., Larrieu, C. and Arnaud, Y. (1988) Rajeunissement *in vitro* chez le *Sequoia sempervirens* (Endl.). *Ann. Rech. Sylvicoles, AFOCEL*, pp 55-81.

Franclet, A., Boulay, M., Bekkaoui, F., Fouret, Y., Verschoore-Martouzet, B. and Walker, N. (1987) Rejuvenation. In: *Forestry Sciences: Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 232-248. Martinus Nijhoff, Dordrecht.

Freire, M.S. and Mumford, P.M. (1986) The efficiency of a range of containers in maintaining seed viability during storage. *Seed Science and Technology* 14: 371-381.

Friedman, J., Rushkin, E. and Waller, G.R. (1982) Highly potent germination inhibitors in *Aqueous eluate* of fruits of Bishop's weed (*Ammi majus* L.) and avoidance of autoinhibition. *Journal of Chemical Ecology* 8(1): 55-65.

Fu, J.R., Zhang, B.Z., Wang, X.P., Qiao, Y.Z. and Huang, X.L. (1990) Physiological studies on desiccation, wet storage and cryopreservation of recalcitrant seeds of three fruit species and their excised embryogenic axes. *Seed Science and Technology* 18: 743-754.

Gaspar, S. and Nagy, J. (1981) A new method for tetrazolium analysis of wheat seeds without embryo excision. *Seed Science and Technology* 9: 553-556.

Gaspar, Th., Kevers, C., Debergh, P., Maene, L., Paques, M. and Boxus, Ph. (1987) Vitrification: morphological physiological and ecological aspects. In: *Forestry Sciences: Cell and Tissue Culture in Forestry, Vol. I.* (eds. J.M. Bonga and D.J. Durzan), pp 152-166. Martinus Nijhoff, Dordrecht.

George, E.F. and Sherrington, P.D. (1984) *Plant propagation by tissue culture: Handbook and Directory of Commercial Laboratories.* Exegetics Ltd, Eversley, Basingstike, Hants, England.

Gladfelter, H.J. and Phillips, G.C. (1987) *De novo* shoot organogenesis of *Pinus eldarica* Medw. *in vitro:* I. Reproducible regeneration from long-term callus cultures. *Plant Cell Reports* 6: 163-166.

Gopal, M. and Thapliyal, R.C. (1969) Topographical tetrazolium test of Indian tree seeds. *Van Vigyan, Dehra Dun* 7: 37-45.

Gorecki, R.J. and Harman, G.E. (1987) Effect of antioxidants on viability and vigour of ageing peas. *Seed Science and Technology* 15: 109-117.

Gosling, P.G. and Rigg, P. (1990) The effect of moisture content and prechill duration on the efficiency of dormancy breakage in Sitka spruce (*Picea sitchensis*) seed. *Seed Science and Technology* 18: 337-343.

Gradwohl, J. and Greenberg, R. (1988) *Saving the tropical forests.* Earthscan Publications Ltd, London.

Grime, J.P. (1981) The role of seed dormancy in vegetation dynamics. *Annals of Applied Biology* 98: 555-558.

Grout, B.W.W., Shelton, K. and Pritchard, H.W. (1983) Orthodox behaviour of oil palm seed and cryopreservation of the excised embryo for genetic conservation. *Annals of Botany* 52: 381-384.

Gupta, P.K. and Grob, J.A. (1995) Somatic embryogenesis in conifers. In: *Somatic Embryogenesis in Woody Plants, Volume 1, History, Molecular and Biochemical Aspects and Applications* (eds. S.M. Jain, P.K. Gupta and R.J. Newton), pp 81-98. Kluwer Academic Publishers, Dordrecht, Boston and London.

Gupta, P.K., Pullman, G., Timmis, R., Kreitinger, M., Carlson, W.C., Grob, J. and Welty, E. (1993) Forestry in the 21st Century: the biotechnology of somatic embryogenesis. *Biotechnology* 11: 454-459.

Haile, B. (1961) Reforestation of exploitation areas (in Ethiopia). *Ethiopian Forestry Review* 1: 17-20.

Hakman, I. and Fowke, L.C. (1987) Somatic embryogenesis in *Picea glauca* (white spruce) and *Picea mariana* (black spruce). *Canadian Journal of Botany* 65: 656-659.

Hakman, I., Fowka, L.C., von Arnold, S. and Eriksson, T. (1985) The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Science* 38: 53-59.

Hanson, J. (1984) the storage of seeds of tropical tree fruits. In: *Crop Genetic Resources: Conservation & Evaluation* (eds. J.H.W. Holden and J.T. Williams), pp 53-62. George Allen & Unwin, London.

Harman, G.E. and Mattick, L.R. (1976) Association of lipid oxidation with seed ageing and death. *Nature* 323-324.

Hartmann, H.T. and Kester, D.E. (1975) *Plant Propagation*. Prentice-Hall, Inc., Engelwood Cliffs, N.J., London.

Harty, R.L. (1983) Testing of tropical species for germination. *Seed Science and Technology* 11: 41-56.

Hendry, G.A.F. (1993) Oxygen, free radical processes and seed longevity. *Seed Science Research* 3: 141-153.

Hewett, P.D. (1987) Pathogen viability on seed in deep freeze storage. *Seed Science and Technology* 15: 73-77.

Heydecker, W. (1977) Stress and seed germination: an agronomic view. In: *The Physiology and Biochemistry of Seed Dormancy and Germination* (ed. A.A. Khan), pp 238-282. North Holland Publishing Company, Amsterdam.

Hilhorst, H.W.M., Smitt, A.I. and Karssen, C.M. (1986) Gibberellin-biosynthesis and sensitivity mediated stimulation of seed germination of *Sisymbrium officinale* by red light and nitrate. *Physiologia Plantarum* 67: 285-290.

Hong, T.D. and Ellis, R.H. (1992a) Optimum air-dry seed storage environments for Arabica coffee. *Seed Science and Technology* 20: 547-560.

Hong, T.D. and Ellis, R.H. (1992b) The survival of germinating orthodox seeds after desiccation and hermetic storage. *Journal of Experimental Botany* 43(247): 239-247.

Hong, T.D. and Ellis, R.H. (1995) A protocol to determine seed storage behaviour. *International Plant Genetic Resources Institute, Rome.*

ISTA (1985) *Handbook on tetrazolium testing*. The International Seed Testing Association, Zurich, Switzerland.

Johns, A.D. (1992) Species conservation in managed tropical forests. *Tropical Deforestation and Species Extinction* (eds. T.C. Whitmore and J.A. Sayer), pp 15-53. Chapman and Hall, London.

Jones, S.K., Samuel, Y.K. and Gosling, P.G. (1991) The effect of soaking and pre-chilling on the germination of noble fir seeds. *Seed Science and Technology* 19: 287-293.

Kalkmann, C. (1965) The old world species of *Prunus* subg. *Laurocerasus* including those formerly referred to *Pygeum*. *Blumea* 13(1): 1-115.

Kariuki, E.M. and Powell, G.R. (1988) Pre-treatment and germination of seeds of three leguminous tree species indigenous to Kenya. *Seed Science and Technology* 16: 477-487.

Kempthorne, O. (1952) *The design and analysis of experiments*. John Wiley and Sons, New York, Chapman and Hall Ltd.

Kendurkar, S.V., Nadgauda, R.S., Phadke, C.H., Jana, M.M., Shirke, S.V. and Mascarenhas, A.F. (1995) Somatic embryogenesis in some woody angiosperms. In: *Embryogenesis in Woody Plants Vol 1* (eds. S.M. Jain, P.K. Gupta and R.J. Newton), pp 49-79. Kluwer Academic Publishers, Dordrecht.

Khan, A.A. (1977) Seed dormancy: changing concepts and theories. In: *The Physiology and Biochemistry of Seed Dormancy and Germination* (ed. A.A. Khan), pp 29-50. North Holland Publishing Company, Amsterdam.

Kimmins, J. P. (1987) *Forest Ecology*. Macmillan Publishing Company, New York.

King, M.W. and Roberts, E.H. (1980) Maintenance of recalcitrant seeds in storage. In: *Recalcitrant Crop Seeds* (eds. H.F. Chin and E.H. Roberts), pp 53-89. Tropical Press, Kuala Lumpur.

Kirby, E.G., Leustek, T. and Lee, M.S. (1987) Nitrogen nutrition. In: *Forestry Sciences: Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 67-88. Martinus Nijhoff, Dordrecht.

Klee, H., Horch, R. and Rogers, S. (1987) Agrobacterium - mediated plant translocation and its further applications to plant biology. *Annual Review of Plant Physiology* 38: 467-486.

Klimaszewska, K., Ward, C. and Cheliak, W.M. (1992) Cryopreservation and plant regeneration from embryogenic cultures of larch (*Larix x eurolepis*) and Black spruce (*Picea mariana*). *Journal of Experimental Botany* 43(246): 73-79.

Konar, R.N., Thomas, E. and Street, H.E. (1972) The diversity of morphogenesis in suspension cultures of *Atropa belladonna* L. *Annals of Botany* 36: 249-258.

Kormode, A.R. and Bewley, J.D. (1988) The role of maturation drying in the translocation from seed development to germination. Responses of the

immature caster bean embryo to isolation from the whole seed: A comparison with premature desiccation. *Journal of Experimental Botany* 39: 487-497.

Koster, K.L. and Leopold, A.C. (1988) Sugars and desiccation-tolerance in seeds. *Plant Physiology* 88: 829-832.

Krogstrup, P. (1989) Micropropagation of juvenile Norway spruce. *Forest Tree Improvement*, No 22, 10 pp. Copenhagen, Denmark.

Kubo, I., Muroi, H., and Himejima, M. (1993) Combination effects of antifungal nagilactones against *Candida albicans* and two other fungi with phenylpropanoids. *Journal of Natural Products (Lloydia)* 56(2): 220-226.

Kumar, P.P., Reid, D.M. and Thorpe, T.A. (1987) The role of ethylene and carbon dioxide in differentiation of shoot buds in excised cotyledons of *Pinus radiata in vitro*. *Physiologia Plantarum* 69: 244-252.

Ladeira, A.M., Gaurdia, M.C. and Takaki, M. (1987) Manipulation of seed germination in *Plantago tomentosa* Lam. and *Raphanus sativus* L. *Seed Science and Technology* 15: 55-63.

Laine, E. and David, A. (1990) Somatic embryogenesis in immature embryos and protoplasts of *Pinus caribaea. Plant Science* 69: 215-224.

Lambardi, M., Sharma, K.K. and Thorpe, T.A. (1991) *In vitro* regeneration of shoot buds and plantlet formation from mature embryo of *Pinus halepensis*. *Acta Horticulturae* 289: 123-124. International Symposium on Plant Biotechnology and its Contribution to Plant Development, Multiplication and Improvement, Geneva, Switzerland.

Leadem, C.L. (1984) Quick tests for tree seed viability. *BC Ministry of Forests, Research Branch as Land Management Report No 18*, Victoria BC, 45pp.

Leadem, C.L. (1995) Effects of stratification and temperature on the germination of *Dalbergia cochinchinensis, Pinus kesiya* and *Pinus mercusii*. *Journal of Tropical Forest Science* 7(3): 355-370.

Leopold, A.C. and Vertucci, C.W. (1986) Physical attributes of desiccated seeds. In: *Membranes, Metabolism and Dry Organisms* (ed. A.C. Leopold), pp 22-34. Comstock Publishing Associates, Ithaca.

Lin, T.P. and Huang, N.H. (1994) The relationship between carbohydrate composition of some tree seeds and their longevity. *Journal of Experimental Botany* 45(278): 1289-1294.

Livesley, M.A. and Bray, C.M. (1991) The effect of ageing upon α–amylase production and protein synthesis by wheat aleuron layers. *Annals of Botany* 68: 69-73.

Longman, K.A. and Jenik, J. (1987) *Tropical forest and its environment*. Longman Scientific & Technical, John Wiley and Sons, New York.

Longo, R. and Tira, S. (1981) Constituents of *Pygeum africanum* bark. *Planta Medica* 42(2): 195-196.

Mantell, S.H., Matthews, J.A. and McKee, R.A. (1985) *Principles of plant biotechnology: An introduction to genetic engineering in plants*. The Alden Press, Osney Mead, Oxford.

Manzanera, J.A. and Pardos, J.A. (1990) Micropropagation of juvenile and adult *Quercus suber*. *Plant Cell, Tissue and Organ Culture* 21(1): 1-8.

Marquis, D.A. (1975) Seed storage and germination under Northern hardwood forest. *Canadian Journal of Forest Research* 5: 478-484.

Martinez-Pulido, C., Harry, I.S., Thorpe, T.A. and Pulido, C.M. (1994) Effect of various bud induction treatments on elongation and rooting of adventitious shoots of Canary Island pine (*Pinus canariensis*). *Plant Cell, Tissue and Organ Culture* 39(3): 225-230.

McCown, B.H. and Sellmer, J.C. (1987) General media and vessels suitable for woody plant culture. In: *Forestry Sciences: Cell and Tissue Culture in Forestry*, Vol. I., eds. J. M. Bonga and D. J. Durzan, 4-6, Martinus Nijhoff, Dordrecht.

McCown, D.D. and McCown, B.H. (1987) North American hardwoods. In: *Case Histories: Gymnosperms, Angiosperms and Palms* (eds. J.J. Bonga and D.J. Durzan), pp 247-260. Cell and Tissue Culture in Forestry, Vol 3, Martinus Nijhoff, Dordrecht.

McKersie, B.D. and Stinson, R.H. (1980) Effect of dehydration on leakage and membrane structure in *Lotus corniculatus* L. seeds. *Plant Physiology* 66: 316-320.

McWilliam, A.A., Smith, S.M. and Street, H.E. (1974) The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). *Annals of Botany* 38: 243-250.

Mesfin, T., Beyenne, S. and Tivell, A. (1987) Trees and shrubs from some hillside enclosures in Wello. SIDA Wello Program, (MOA)/Upper Mille Cheleka Catchment Disaster Prevention Program, Addis Ababa, Ethiopia, vii + 108pp.

Michaux-Ferriére, N., Grout, H. and Carron, M.P. (1992) Origin and ontogenesis of somatic embryos in *hevea brasiliensis* (Euphorbiaceae). *American Journal of Botany* 79(2): 174-180.

Minocha, S.C. (1987a) pH of the medium and growth and metabolism of cells in culture. In: *Forestry Sciences: Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 125-141. Martinus Nijhoff, Dordrecht.

Minocha, S.C. (1987b) Plant growth regulators and morphogenesis in cell and tissue culture of forest trees. In: *Forestry Sciences: Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 50-66. Martinus Nijhoff, Dordrecht.

Misra, S., Attree, S.M., Leal, I. and Fowke, L.C. (1993) Effect of abscisic acid, osmoticum and desiccation on synthesis of storage proteins during the development of White spruce somatic embryos. *Annals of Botany* 71: 11-22.

Mo, L.H. and von Arnold, S. (1991) Origin and development of embryogenic cultures from seedling of Norway spruce (*Picea abies*). *Journal of Plant Physiology* 138: 223-230.

Montagnini, F. and Sancho, F. (1990) Impacts of native trees on tropical soils: A study in the Atlantic lowlands of Costa Rica. *Ambio* 19: 386-390.

Montoro, P., Etienne, H., Michaux-Ferriére, N. and Carron, M.P. (1993) Callus friability and somatic embryogenesis in *hevea brasiliensis*. *Plant Cell, Tissue and Organ Culture* 33: 331-338.

Moore, R.P. (1985) *Handbook on tetrazolium testing.* International Seed Testing Association, Zurich, Switzerland.

Muller, C. and Bonnet-Masimbert, M. (1989) Breaking dormancy before storage: an improvement to processing of beechnuts (*Fagus sylvatica*). *Seed Science and Technology* 17: 15-26.

Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bio-assays with tobacco tissue culture. *Physiol. Plant.* 15: 473-497.

Nagmani, R., Diner, A.M. and Sharma, G.C. (1993) Somatic embryogenesis in long leaf pine (*Pinus palustris*). *Canadian Journal of Research* 23: 873-876.

Negussie, A., Sood, J. E. and Mayhead, G. J. (1991) The effect of pre-treatments and diurnal temperature variations on the germination of *Juniperus excelsa. The International Tree Crop Journal* 7: 57-66.

Neuman, M.C., Preece, J.E., Van Sambeek, J.W. and Gaffney, G.R. (1993) Somatic embryogenesis and callus production from cotyledon explants of Eastern black walnut. *Plant Cell, Tissue and Organ Culture* 32: 9-18.

Nkang, A. (1988) Some aspects of the biochemical basis of viability loss in stored *Guilfoylia monostylis* seeds. *Seed Science and Technology* 16: 247-260.

Nkang, A. and Chandler, G. (1986) Changes during embryogenesis in rain forest seeds with orthodox and recalcitrant viability characteristics. *Journal of Plant Physiology* 126: 243-256.

Noel, A.R.A. and Staden, J.V. (1976) Seed coat structure and germination in *Podocarpus henkelii. Zeitschrift Fur Pflanzenphysiologie* 77: 174- 186.

Noh, E.W., Minocha, S.C. and Riemenschneider, D.E. (1988) Adventitious shoot formation from embryonic explants of red pine (*Pinus resinosa*). *Physiologia Plantarum* 74: 119-124.

O'Dowd, T. and Dobie, P. (1983) Reducing viability losses in open seed stores in tropical climates. *Seed Science and Technology* 11: 57-75.

Ogata, K. (1991) Useful timbers in the tropics. *Tropical Forestry* 20: 50-51.

Ojera, H. and Trione, S.O. (1990) Effect of high temperatures on germination of guayule seeds. *Seed Science and Technology* 18: 681-691.

Okusanya, O.T. and Ungar, I.A. (1983) The effect of time of seed production on the germination response of *Spergularia marina*. *Physiologia Plantarum* 59: 335-342.

Overaa, P. (1979) Introduction to the biochemical test for viability programme. *Seed Science and Technology* 7: 591-596.

Page, C.N. (1988) New and maintained genera in the conifer families *Podocarpaceae* and *Pinaceae*. *Notes RBG Edinburgh* 45(2): 377-395.

Pammenter, N.W., Vertucci, C.W. and Berjak, P. (1991) Homeohydrous (recalcitrant) seeds: Dehydration, the state of water and viability characteristics in *Landolphia Kirkii*. *Plant Physiology* 96: 1093-1098.

Pandey, D.K. (1989) Short duration accelerated ageing of French bean seeds in hot water. *Seed Science and Technology* 17: 107-114.

Parfitt, D.E. and Arulsekar, S. (1987) Measurement and origin of genetic variation in tissue culture systems. In: *Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 286-297. Martinus Nijhoff, Dordrecht.

Park, Y.G. and Son, S.H. (1988) *In vitro* organogenesis and somatic embryogenesis from punctured leaf of *Populus nigra* x *P. maximowiezii*. *Plant Cell, Tissue and Organ Culture* 15: 95-105.

Pereira, M.F.A. and Maeda, J.A. (1986) Environmental and endogenous control of germination of *Vitis vinifera* seeds. *Seed Science and Technology* 14: 227-235.

Perez-Parron, M.A., Gonzalez-Benito, M.E. and Perez, C. (1994) Micropropagation of *Fraxinus augustifolia* from mature and juvenile plant material. *Plant Cell, Tissue and Organ Culture* 37(3): 297-302.

Perl, M. (1988) Recent developments in seed physiology and biochemistry. *Seed Science and Technology* 16: 135-138.

Pevalek-Kozlina, B. and Kelaska, S. (1989) Adventitious root formation in cloned microcuttings of *Prunus avium*. *Bioloski-Vestnik* 37(3): 57-66.

Pohjonen, V. and Pukkala, T. (1990) *Eucalyptus globulus* in Ethiopian forestry. *Forest Ecology and Management* 36: 19-31.

Ponnuswamy, A.S., Chellapillai, K.A., Vinayarai, R.S. and Surendran, C. (1991) Effect of collection date and hydration-dehydration treatment on seed viability and vigour of *Ailanthus excelsa,* Roxb. *Seed Science Technology* 19: 591-595.

Pons, T.L. (1992) Seed responses to light. In: *Seeds: The Ecology of Regeneration in Plant Communities* (ed. M. Fenner), pp 285-325. CAB International, Wallingford, Oxon.

Pritchard, H.W., Wood, J.A. and Manger, K.R. (1993) Influence of temperature on seed germination and the nutritional requirements for embryo growth in *Arum maculatum* L. *New Phytologist* 123: 801-809.

Purohit, S.D., Kukda, G., Sharma, P. and Tak, K. (1994) *In vitro* propagation of an adult tree *Wrightia tomentosa* through enhanced axillary branching. *Plant Science Limerick* 103(1): 67-72.

Quinlivan, B.J., Collins, W.J. and Vistisen, M.I. (1987) The effect of tempeature on the rate of seed development in subterranean clover. *Seed Science and Technology* 15: 633-638.

Radojević, L., Vujicić, R. and Nesković (1975) Embryogenesis in tissue culture of *Corylus avellana* L. *Z. Pflanzenphysiol. Bd.* 77: 33-41.

Rai, V.R. and Chandra, K.S.J. (1988) *In vitro* regeneration of plantlets from shoot callus of mature tree of *Dalbergia latifolia. Plant Cell, Tissue and Organ Culture* 13: 77-83.

Roberts, D.R., Sutton, B.C.S. and Flinn, B.S. (1990) Synchronous and high frequency germination of interior spruce somatic embryos following partial drying at high relative humidity. *Canadian Journal of Botany* 68: 1086-1090.

Roberts, E.H. (1973) Loss of viability: ultrastructural and physiological aspects. *Seed Science and Technology* 1: 529-545.

Roberts, E.H. and Ellis, R.H. (1984) The implications of the deterioration of orthodox seeds during storage for genetic resources conservation. In: *Crop Genetic Resources: Conservation and Evaluation* (eds. J.H.W. Holden and J.T. Williams), pp 18-37. George Allen & Unwin, London.

Roberts, E.H.and Ellis, R.H. (1989) Water and seed survival. *Annals of Botany* 63: 39-52.

Roberts, E.H. and King, M.W. (1980) Storage of recalcitrant seeds. In: *Crop Genetic Resources: The Conservation of Difficult Material* (eds. L.A. Withers and J.T. Williams), pp 39-48. Proceedings of an International Workshop, University of Reading, UK.

Roberts, E.H., King, M.W. and Ellis, R.H. (1984) Recalcitrant seeds: their recognition and storage. In: *Crop Genetic Resources: Conservation and Evaluation* (eds. J.H.W. Holden and J.T. Williams), pp 38-52. George Allen & Unwin, London.

Romano, A., Noronha, C. and Martins-Loucas, M.A. (1992) Influence of growth regulators on shoot proliferation in *Quercus suber* L. *Annals of Botany* 70(6): 531-536.

Roy, M.M., Pathak, P.S. and Roy, R.D. (1984) Seed scarification requirement in *Dichrostachys cinerea* (L.) Wight and Arn. *Journal of Tree Sciences* 3: 143-145.

Roy, S.K., Islam, M.S., Sen, J., Hossain, A.B.M.E. and Hadiuzzaman, S. (1993) Propagation of flood tolerant jackfruit (*Artocarpus heterophyllus*) by *in vitro* culture. *Acta-Horticulture* 336: 273-278. Second International Symposium, Baltimore, USA.

Ruredzo, T.J. and Hanson, J. (1993) Plant recovery from seedling-derived shoot tips of *Faidherbia albida* grown *in vitro*. *Agroforestry Systems* 22(1): 59-65.

Sahlén, K. and Gjelsvik, S. (1993) Determination of *Pinus sylvestris* seed maturity using leachate conductivity measurements. *Canadian Journal of Forest Research* 23: 864-870.

Salajova, T. (1993) Adventitious bud development *in vitro* on mature zygotic embryos of *Pinus nigra* Arn. *Biologia Bratislava* 48(1): 105-108.

Saulei, S.M. (1984) Natural regeneration following clear-fell logging operations in the Gogol Valley, Papua New Guinea. *Ambio* 13: 351-354.

Schuller, A., Reuther, G. and Geier, T. (1989) Somatic embryogenesis from seed explants of *Abies alba*. *Plant Cell, Tissue and Organ Culture* 17: 53-58.

Scott, E.S., Rao, A.N. and Loh, C.S. (1988) Production of plantlets of *Shorea roxburghii* G. Don. from embryogenic axes cultured *in vitro*. *Annals of Botany* 61: 233-236.

Sengi, Y. (1990) *In vitro* plantlet regeneration from axillary buds of an adult mizunara (*Quercus crispula*). *Journal of the Japanese Forestry Society* 72(2): 143-146.

Sevilla, E.P. (1987) Germination and tetrazolium testing. *Seed Science and Technology* 15: 691-698.

Shaefer, C. (1990) Seed testing research on species indigenous to Kenya. *Proceedings of an International Workshop, Forestry Training Centre, Gympie, Queensland, Australia, 21-24 August 1989* (ed. J.W. Turnbull). ACIAR Proceedings Series, 1990, No 28, pp 132-139.

Sharma, S. and Chandra, N. (1988) Organogenesis and plantlet formation *in vitro* in *Dalbergia sissoo* Roxb. *Journal of Plant Physiology* 132: 145-147.

Siegel, S. (1956) *Kruskal-Wallis Test, Non-Parametric Statistics for the Behavioural Sciences*. McGraw Hill, p 184.

Simak, M. (1970) Germination analysis of *Abies alba* seed. *Proceedings of International Seed Testing Association* 35(2): 361-367.

Söndahl, M.R. and Sharp, W.R. (1977) High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. *Zeitschrift Fur Pflanzenphysiologie* 81: 395-408.

Spears, J.S. (1981) Small farmers - or the tropical forest ecosystem? A review of sustainable land use systems for tropical forest areas. In: *Tropical Forests-utilisation and Conservation* (ed. F. Mergen), pp 14-47. Yale University, New Haven, CT.

Steward, F.C., Mapes, M.O. and Mears, K. (1958) Growth and organised deelopment of cultured cells. II. Organisation in cultures grown from freely suspended cells. *American Journal of Botany* 45: 705-708.

Tacon, F.L. and Harley, J.L. (1990) Deforestation in the tropics and the proposals to arrest it. *Ambio* 19: 372-378.

Tautorus, T.E., Fowke, L.C. and Dunstan, D.I. (1991) Somatic embryogenesis in conifers. *Canadian Journal of Botany* 69: 1873- 1899.

Thanos, C.A. and Skordilis, A. (1987) The effect of light, temperature and osmotic stress on the germination of *Pinus halepensis* and *P. brlltia* seeds. *Seed Science and Technology* 15: 163- 174.

Thapliyal, P. and Nautiyal, A.R. (1989) Imbibition of seed germination by pericarp in *Fraxinus micrantha* Lang. *Seed Science and Technology* 17: 125-130.

Thapliyal, R.C., Sood, O.P. and Rawat, M.M.S. (1991) Effect of moisture content and storage temperature on the viability of *Bambusa tulda* seeds. *The International Tree Crops Journal* 7: 67-75.

Thompson, K. (1992) The functional ecology of seed banks. In: *Seeds: The Ecology of Rengeration in Plant Communities* (ed. M. Fenner), pp 231-258. CAB International, Wallingford, Oxon.

Thompson, M.R. and Thorpe, T.A. (1987) Metabolic and non-metabolic roles of carbohydrates. In: *Forestry Sciences: Cell and Tissue Culture in Forestry, vol. I* (eds. J.M. Bonga and D.J. Durzan), pp 89-112. Martinus Nijhoff, Dondrecht.

Thornhill, A. and Koopowitz, H. (1992) Viability of *Disa uniflora* Berg (*Orchidaceae*) seeds under variable storage conditions: Is orchid gene-banking possible? *Biological Conservation* 62: 21-27.

Thorpe, T.A., Harry, I.S. and Kumar, P.P. (1991) Application of micropropagation to forestry. In: *Micropropagation* (eds. P.C. Debergh and R.H. Zimmerman), pp 311-336. Kluwer Academic Publisher, Netherlands.

Tilden, R.L. and West, S.H. (1985) Reversal of the effects of ageing in soybean seeds. *Plant Physiology* 77: 584-586.

Timmis, R., Abo El-Nil, M.M. and Stonesypher, R.W. (1987) Potential genetic gain through tissue culture. In: *Cell and Tissue Culture in Forestry* (eds. J.M. Bonga and D.J. Durzan), pp 198-215. Martinus Nijhoff, Dordrecht.

Tomar, U.K. and Gupta, S.C. (1988) *In vitro* plant regeneration of leguminous trees (*Albizia* spp). *Plant Cell Reports* 7(6): 385-388.

Tompsett, P.B. (1984) The effect of moisture content and temperature on the seed storage life of *Araucaria columnaris*. *Seed Science and Technology* 12: 801-816.

Tompsett, P.B. (1992) A review of the literature on storage of dipterocarp seeds. *Seed Science and Technology* 20: 251-267.

Tremblay, F.M. (1990) Somatic embryogenesis and plantlet regeneration from embryos isolated from stored seeds of *Picea glauca*. *Canadian Journal of Botany* 68: 236-241.

Tulecke, W. and McGranaham, G. (1985) Somatic embryogenesis and plant regeneration from cotyledons of walnut, *Juglans regia* L. *Plant Science* 40: 57-63.

Verhagen, S.A. and Wann, S.R. (1989) Norway spruce somatic embryogenesis: high-frequency initiation from light-cultured mature embryos. *Plant Cell, Tissue and Organ Culture* 16: 103-111.

Vertucci, C.W. and Leopold, A.L. (1986) Physiological activities associated with hydration level in seeds. In: *Membranes, Metabolism and Dry Organisms* (ed. A.C. Leopold), pp 35-49. Comstock Publishing Associates, Ithaca.

Villiers, T.A. and Edgcumbe, D.J. (1975) On the cause of seed deterioration in dry storage. *Seed Science and Technology* 3: 761-774.

Volkaert, H., Schoofs, J., Pieters, A. and De-Langhe, E. (1990) Influence of explant source on *in vitro* axillary shoot formation in oak seedlings. *Tree Physiology* 6(1): 87-93.

von Arnold, S. and Hakman, I. (1988) Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA). *Journal of Plant Physiology* 132: 164-169.

Vossen van der, H.A.M. (1979) Methods of preserving the viability of coffee seed in storage. *Seed Science and Technology* 7: 65-74.

Wainwright, H. and Tutt, D.E. (1987) Quick concentrated auxin dips for rooting birch. *Advances in Horticultural Science* 1(2): 87-89.

Wang, B.S.P. (1988) Review of new development in tree seeds. *Seed Science and Technology* 16: 215-225.

Wann, S.R. (1989) Somatic embryogenesis in woody species. In: *Horticultural Reviews Vol 10* (ed. J. Janick), pp 153-181. Timber, Portland, Oregon.

Warham, E.J. (1986) A comparison of packing materials for seed with particular reference to humid tropical environments. *Seed Science and Technology* 14: 191-211.

Westoby, J. (1989) *Introduction to world forestry.* Basil Blackwell Ltd., Oxford, UK.

Whitmore, T.C and Sayer, J.A. (1992) Deforestation and species extinction in tropical moist forest. In: *Tropical Deforestation and Species Extinction* (eds. T.C. Whitmore and J.A. Sayer), pp 1-14. Chapman and Hall, London.

Williams, E.G. and Maheswaran, G. (1986) Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group - Review Article. *Annals of Botany* 57: 443-462.

World Resources Institute (1990) *World resources 1990-91.* Oxford University Press.

APPENDIX 3.1a

Rank of mean viability (probit scale), withdrawal 1

MC %	Temp °C	Probit mean	Rank															
8	10	0.65	a															
11	10	0.65	a															
11	21	0.65	a															
6	10	0.60	a	b														
8	21	0.59	a	b	c													
8	4	0.58	a	b	c	d												
15	21	0.58	a	b	c	d												
6	4	0.47	a	b	c	d	e											
11	4	0.47	a	b	c	d	e											
15	10	0.47	a	b	c	d	e											
6	21	0.47	a	b	c	d	e											
15	4	0.41	a	b	c	d	e	f										
6	-20	0.20						f	g									
8	-20	0.20						f	g									
11	-20	0.10							g	h								
15	-20	-0.41									i							
LSD = 0.26 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
8		0.50	a					
11		0.47	a	b				
6		0.44	a	b	c			
15		0.26				d		
	10	0.59	a					
	21	0.57	a	b				
	4	0.48	a	b	c			
	-20	0.02				d		
LSD = 0.13 Total mean								

APPENDIX 3.1b

Rank of mean viability (probit scale), withdrawal 2

MC %	Temp °C	Probit mean	Rank															
8	4	0.53	a															
8	10	0.47	a	b														
6	4	0.47	a	b														
15	4	0.42	a	b	c													
6	10	0.41	a	b	c	d												
11	4	0.41	a	b	c	d												
11	10	0.36	a	b	c	d	e											
8	21	0.15					e	f										
6	21	0.10						f	g									
8	-20	0.05						f	g	h								
6	-20	0.00						f	g	h	i							
11	-20	0.00						f	g	h	i							
15	10	0.00						f	g	h	i							
11	21	-0.10							g	h	i	j						
15	21	-0.53											k					
15	-20	-0.65											k	l				
LSD = 0.21 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
8		0.30	a					
6		0.25	a	b				
11		0.17		b	c			
15		-0.19				d		
	4	0.46	a					
	10	0.31		b				
	21	-0.09			c			
	-20	-0.15			c	d		
LSD = 0.11 Total mean								

APPENDIX 3.1c

Rank of mean viability (probit scale), withdrawal 3

MC %	Temp °C	Probit mean	Rank														
6	10	0.53	a														
8	10	0.47	a	b													
8	4	0.46	a	b	c												
6	4	0.41	a	b	c	d											
15	4	0.26	a	b	c	d	e										
11	10	0.25	a	b	c	d	e	f									
11	4	0.21	a	b	c	d	e	f	g								
6	21	0.20		b	c	d	e	f	g	h							
6	-20	0.05					e	f	g	h	i						
8	21	0.00					e	f	g	h	i	j					
8	-20	-0.05					e	f	g	h	i	j	k				
15	10	-0.15									i	j	k	l			
11	-20	-0.31										j	k	l	m		
11	21	-0.36											k	l	m	n	
15	-20	-1.58															o
15	21	-1.75															o p
LSD = 0.32 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		0.30	a					
8		0.22	a	b				
11		-0.05			c			
15		-0.81				d		
	4	0.34	a					
	10	0.28	a	b				
	-20	-0.47			c			
	21	-0.48			c	d		
LSD = 0.16 Total mean								

APPENDIX 3.1d

Rank of mean viability (probit scale), withdrawal 4

MC %	Temp °C	Probit mean	Rank															
8	10	0.53	a															
8	4	0.47	a	b														
6	4	0.41	a	b	c													
6	10	0.41	a	b	c													
11	4	0.26		b	c	d												
11	10	0.26		b	c	d												
6	21	0.10				d	e											
15	4	0.05				d	e	f										
8	21	-0.05					e	f	g									
6	-20	-0.10					e	f	g	h								
8	-20	-0.20							g	h	i							
11	-20	-0.41									i	j						
11	21	-0.47										j	k					
15	20	-0.59										j	k	l				
15	-20	-1.58													m			
15	21	-1.75													m	n		
LSD = 0.23 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
6		0.21	a					
8		0.19	a	b				
11		-0.09			c			
15		-0.97				d		
	4	0.30	a					
	10	0.15		b				
	21	-0.54			c			
	-20	-0.57			c	d		
LSD = 0.12 Total mean								

APPENDIX 3.2

Rank of slopes for withdrawals 0, 1, 2, 3 and 4

MC %	Temp °C	Slope (probit)	Rank														
8	10	-0.57	a														
8	4	-0.62	a	b													
6	4	-0.69	a	b	c												
6	10	-0.70	a	b	c	d											
11	4	-1.26					e										
11	10	-1.40					e	f									
6	21	-1.60					e	f	g								
15	4	-1.60					e	f	g								
6	-20	-1.92						f	g	h							
8	-20	-2.25								h	i						
8	21	-2.28								h	i	j					
11	-20	-2.88											k				
15	10	-3.51												l			
11	21	-3.66												l	m		
15	-20	-6.27														n	
15	21	-7.93															o
LSD = 0.53 MC*Temp interaction																	

MC %	Temp °C	Slope (probit)	Rank					
6		-1.23	a					
8		-1.43	a	b				
11		-2.30			c			
15		-4.83				d		
	4	-1.04	a					
	10	-1.54		b				
	-20	-3.33			c			
	21	-3.87				d		
LSD = 0.26 Total mean								

APPENDIX 4.1a

Rank of mean viability (probit scale) for the effect of moisture content and temperature interaction and for the total means.

WD1

MC %	Temp °C	Probit mean	Rank														
15	4	0.81	a														
15	21	0.76	a	b													
11	10	0.50	a	b	c												
15	10	0.44	a	b	c	d											
6	21	0.35		b	c	d	e										
8	4	0.35		b	c	d	e										
6	4	0.32			c	d	e	f									
8	10	0.26			c	d	e	f	g								
11	4	0.14			c	d	e	f	g	h							
6	10	0.09			c	d	e	f	g	h	i						
8	-20	0.00					e	f	g	h	i	j					
6	-20	-0.10						f	g	h	i	j	k				
11	-20	-0.15							g	h	i	j	k	l			
8	21	-0.21								h	i	j	k	l	m		
11	21	-0.50											k	l	m	n	
15	-20	-0.87														n	o
LSD = 0.42 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
15		0.29	a					
6		0.17	a	b				
8		0.10	a	b	c			
11		-0.00		b	c	d		
	4	0.41	a					
	10	0.32	a	b				
	21	0.10			c			
	-20	-0.28				d		
LSD = 0.21 Total mean								

APPENDIX 4.1b

Rank of mean viability (probit scale) for the effect of moisture content and temperature interaction and for the total means.

WD2

MC %	Temp °C	Probit mean	Rank														
15	4	0.29	a														
6	4	0.09	a	b													
8	4	0.08	a	b	c												
11	4	0.01	a	b	c	d											
11	10	0.01	a	b	c	d											
6	10	0.00		b	c	d	e										
15	10	-0.07		b	c	d	e	f									
6	-20	-0.14		b	c	d	e	f	g								
8	21	-0.23				d	e	f	g	h							
11	21	-0.24				d	e	f	g	h	i						
6	21	-0.28					e	f	g	h	i	j					
8	10	-0.44								h	i	j	k				
11	-20	-0.49								h	i	j	k	l			
8	-20	-0.52									i	j	k	l	m		
15	-20	-1.19														n	
15	21	-1.19														n	
LSD = 0.28 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		-0.08	a					
11		-0.17	a	b				
8		-0.28		b	c			
15		-0.54				d		
	4	0.12	a					
	10	-0.12		b				
	21	-0.48			c			
	-20	-0.58			c	d		
LSD = 0.14 Total mean								

APPENDIX 4.1c

Rank of mean viability (probit scale) for the effect of moisture content and temperature interaction and for the total means.

WD3

MC %	Temp °C	Probit mean	Rank															
8	4	0.21	a															
6	4	0.14	a	b														
11	4	0.08	a	b	c													
15	4	0.08	a	b	c													
6	21	0.05	a	b	c	d												
8	21	0.00		b	c	d	e											
11	10	0.00		b	c	d	e											
6	10	-0.02		b	c	d	e	f										
8	10	-0.02		b	c	d	e	f										
15	10	-0.24							g									
11	21	-0.27							g	h								
6	-20	-0.37							g	h	i							
8	-20	-0.42							g	h	i	j						
11	-20	-0.44							g	h	i	j	k					
15	21	-1.06												l				
15	-20	-1.19												l	m			
LSD = 0.20 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
6		-0.05	a					
8		-0.06	a	b				
11		-0.16		b	c			
15		-0.60				d		
	4	0.13	a					
	10	-0.07		b				
	21	-0.32			c			
	-20	-0.60				d		
LSD = 0.10 Total mean								

APPENDIX 4.1d

Rank of mean viability (probit scale) for the effect of moisture content and temperature interaction and for the total means.

WD4

MC %	Temp °C	Probit mean	Rank															
6	10	-0.02	a															
8	21	-0.07	a	b														
8	4	-0.10	a	b	c													
15	4	-0.12	a	b	c	d												
8	10	-0.15	a	b	c	d	e											
11	4	-0.20		b	c	d	e	f										
6	4	-0.20		b	c	d	e	f										
6	-20	-0.29				d	e	f	g									
6	21	-0.37						f	g	h								
11	-20	-0.37						f	g	h								
11	10	-0.38							g	h	i							
8	-20	-0.43							g	h	i	j						
11	21	-0.71											k					
15	10	-0.91												l				
15	-20	-2.07													m			
15	21	-2.07													m			
LSD = 0.17 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
8		-0.19	a					
6		-0.22	a	b				
11		-0.29		b	c			
15		-0.41				d		
	4	-0.15	a					
	10	-0.36		b				
	-20	-0.79			c			
	21	-0.81			c	d		
LSD = 0.08 Total mean								

APPENDIX 4.2

Rank of mean slope for the effect of moisture content and temperature interaction and for the total means: withdrawals 0, 1, 2, 3 and 4.

MC %	Temp °C	Slope (probit)	Rank															
8	21	-1.47	a															
6	10	-1.72	a	b														
8	4	-1.92	a	b	c													
11	4	-2.04	a	b	c	d												
8	10	-2.17		b	c	d	e											
6	4	-2.19		b	c	d	e	f										
6	-20	-2.48			c	d	e	f	g									
15	4	-2.62				d	e	f	g	h								
11	-20	-2.68					e	f	g	h	i							
6	21	-2.70					e	f	g	h	i	j						
11	21	-2.87							g	h	i	j	k					
11	10	-2.92							g	h	i	j	k	l				
8	-20	-2.94							g	h	i	j	k	l	m			
15	10	-4.30														n		
15	-20	-6.45															o	
15	21	-8.11																p
LSD = 0.60 MC*Temp interaction																		

MC %	Temp °C	Probit mean	Rank					
8		-2.12	a					
6		-2.27	a	b				
11		-2.63			c			
15		-5.37				d		
	4	-2.19	a					
	10	-2.78		b				
	-20	-3.63			c			
	21	-3.78			c	d		
LSD = 0.30 Total mean								

APPENDIX 5.1a

Viability rank between means using LSD

WD1

MC %	Temp °C	Probit mean	Rank														
6	4	-.29	a														
8	4	0.27	a	b													
8	10	0.25	a	b	c												
6	-5	0.24	a	b	c	d											
6	10	0.22	a	b	c	d	e										
8	-5	0.22	a	b	c	d	e										
10	10	0.20	a	b	c	d	e	f									
10	4	0.12		b	c	d	e	f	g								
10	-5	0.02							g	h							
17	4	-0.02							g	h	i						
6	21	-0.05								h	i	j					
8	21	-0.17									i	j	k				
17	-5	-0.20										j	k	l			
10	21	-0.20										j	k	l			
17	10	-0.22											k	l	m		
17	21	-0.42														n	
LSD = 0.16 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		0.18	a					
8		0.14	a	b				
10		0.04			c			
17		-0.22				d		
	4	0.17	a					
	10	0.11	a	b				
	-5	0.07		b	c			
	21	-0.21				d		
LSD = 0.08 Total mean								

APPENDIX 5.1b

Viability rank between means using LSD

WD2

MC %	Temp °C	Probit mean	Rank														
6	4	0.22	a														
8	4	0.22	a														
8	10	0.22	a														
6	-5	0.19	a	b													
6	10	0.17	a	b	c												
10	10	0.15	a	b	c	d											
8	-5	0.07	a	b	c	d	e										
10	4	-0.02		b	c	d	e	f									
10	-5	-0.10					e	f	g								
17	4	-0.30							g	h							
6	21	-0.53									i						
17	-5	-0.62									i	j					
8	21	-0.65									i	j	k				
10	21	-0.70									i	j	k	l			
17	10	-0.92													m		
17	21	-1.32														n	
LSD = 0.21 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		0.01	a					
8		-0.03	a	b				
10		-0.16			c			
17		-0.79				d		
	4	0.03	a					
	10	-0.10		b				
	-5	-0.11		b	c			
	21	-0.80				d		
LSD = 0.11 Total mean								

APPENDIX 5.1c

Viability rank between means using LSD

WD3

MC %	Temp °C	Probit mean	Rank														
6	4	0.07	a														
6	-5	-0.15		b													
8	10	-0.15		b													
8	-5	-0.20		b	c												
8	4	-0.22		b	c	d											
6	10	-0.22		b	c	d											
10	4	-0.22		b	c	d											
10	10	-0.51					e										
17	4	-0.53					e	f									
6	21	-0.56					e	f	g								
10	-5	-0.59					e	f	g	h							
8	21	-0.88									i						
17	-5	-0.91									i	j					
17	10	-1.16											k				
10	21	-1.20											k	l			
17	21	-1.95													m		
LSD = 0.19 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		-0.21	a					
8		-0.36		b				
10		-0.63			c			
17		-1.14				d		
	4	-0.23	a					
	-5	-0.46		b				
	10	-0.51		b	c			
	21	-1.15				d		
LSD = 0.10 Total mean								

APPENDIX 5.1d

Viability rank between means using LSD

WD4

MC %	Temp °C	Probit mean	Rank														
6	4	-0.66	a														
6	10	-0.79	a	b													
8	21	-1.07		b	c												
10	10	-1.23			c	d											
8	-5	-1.25			c	d	e										
6	21	-1.32			c	d	e	f									
10	-5	-1.32			c	d	e	f									
8	10	-1.38			c	d	e	f	g								
17	4	-1.63						f	g	h							
8	4	-1.75								h	i						
10	4	-1.75								h	i						
6	-5	-1.82								h	i	j					
10	21	-1.82								h	i	j					
17	-5	-1.95								h	i	j	k				
17	10	-2.07									i	j	k	l			
17	21	-2.07									i	j	k	l			
LSD = 0.36 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		-1.15	a					
8		-1.35		b				
10		-1.53		b	c			
17		-1.93				d		
	10	-1.37	a					
	4	-1.44	a	b				
	21	-1.57		b	c			
	-5	-1.58		b	c	d		
LSD = 0.18 Total mean								

APPENDIX 5.2

Ranking of mean slopes for withdrawals 0, 1, 2, 3 and 4

MC %	Temp °C	Probit mean	Rank														
6	4	-2.50	a														
6	10	-3.02	a	b													
8	21	-3.91			c												
8	-5	-4.01			c	d											
6	21	-4.27			c	d	e										
10	10	-4.28			c	d	e	f									
8	10	-4.30			c	d	e	f	g								
10	-5	-4.38			c	d	e	f	g	h							
17	4	-4.95					e	f	g	h	i						
10	4	-5.05					e	f	g	h	i	j					
8	4	-5.09					e	f	g	h	i	j	k				
6	-5	-5.26									i	j	k	l			
17	-5	-5.87											k	l	m		
10	21	-5.90											k	l	m	n	
17	10	-6.38													m	n	o
17	21	-6.99															o	p
LSD = 0.82 MC*Temp interaction																	

MC %	Temp °C	Probit mean	Rank					
6		-3.76	a					
8		-4.33		b				
10		-4.90			c			
17		-6.05				d		
	4	-4.40	a					
	10	-4.50	a	b				
	-5	-4.88		b	c			
	21	-5.27			c	d		
LSD = 0.41 Total mean								

APPENDIX 6.1

Seed Pre-treatment and Surface Sterilisation

The seeds were transported in an open vehicle from their collection sites 10-15 days after collection to the Forestry Research Centre, Addis Ababa. They were soaked in water for about 2-3 days in order to soften the external dried fleshy tissues. The pulps were then removed by hand and discarded. The seeds were dried in the sun for 2-5 days to a moisture content of 10-13%. They were stored in the Centre at about 10 °C until they were transported to Edinburgh by air.

The seed coats were cracked in a vice, carefully removed by hand and discarded. The gametophytes containing the embryos were soaked overnight in distilled water. These explants were surface sterilised by stirring for 20 minutes in 0.3% mercuric chloride with two drops of Tween-80. The plant tissues were washed five times each with sterile distilled water and stored in sterile distilled water until they were transferred to the required media.

APPENDIX 6.2

Preparation of MS Basal Medium

The required volume of distilled water was measured into a stainless steel pot. Full-strength MS basal salts and vitamins (Sigma) and 3% sucrose were added and stirred until they dissolved. The pH of the solution was adjusted to 5.5. The solution was heated to boiling without stirring. Bacteriological Agar No 1 (Oxoid) was added at a concentration of 1% and the whole solution was stirred until the agar was fully hydrated. The medium was dispensed in 15 ml aliquots into 10 × 2.5 cm flat bottomed soda glass tubes and capped with aluminium caps. The tubes of medium were autoclaved at 121 °C for 20 minutes, allowed to gel and stored at 2 °C.

APPENDIX 6.3

Sterilisation of germinating shoots with calcium hypochlorite

One hundred and thirty five explants (shoots) from germinating A2 seed lot were categorised into 3, based on their sizes as follows: (1) those explants with less than 2 cm height were categorised as Category A (60 explants); (2) those explants with the height between 2 and 4 cm as Category B (60 explants); and (3) those with the height above 4 cm as Category C (15 explants). Each category was subdivided into three groups of sterilisation time (5, 10 and 15) minutes. Twenty explants were selected randomly for each treatment time for Category A and B, and five explants for Category C.

The root portion of the explants was discarded and the shoots were washed twice with distilled water in honey jars by gently shaking for one minute each. One group in each category was selected randomly and sterilised with 7% calcium hypochlorite with two drops of Tween-80 by shaking for 5 minutes. The second group was also treated in the same way but for 10 minutes, and the third group for 15 minutes. The calcium hypochlorite was discarded after each sterilisation and the explants were washed with sterile distilled water by shaking five times for five minutes each and stored in sterile distilled water until they were transferred to the culture tubes in the laminar flow cabinet.

APPENDIX 6.4

Sterilisation of germinating shoots with mercuric chloride

One hundred and forty one germinating shoots were categorised into two, A and B. Explants with the height of below 2 cm were categorised as A, and the remaining explants above 2 cm categorised as B. The number of explants sorted out for A in this case was 33, and the remaining 108 explants were allocated to B. Each category of A and B was subdivided into three groups based on the sterilisation time of 1, 2 and 3 minutes. A group from each category was treated either for 1, 2 or 3 minutes after random selection. Hence, 11 explants were in each group of A, and 36 explants in each group of B.

The root parts of the explants were discarded and the shoots were washed twice in distilled water in honey jars by gently shaking for one minute each. The explants in each group were sterilised with 0.3% mercuric chloride with two drops of Tween-80 for the required sterilisation time by shaking the jar gently. The mercuric chloride was discarded and the shoots were washed five times with sterile distilled water by gently shaking for 5 minutes. The explants were stored in sterile distilled water until transfer to culture in the laminar flow cabinet.

APPENDIX 7.1

Preparation of media containing hormones and glutamine

The required volume of distilled water was measured into a stainless steel pot. The required amount of basal salts (e.g. 2.3 g/ℓ WPM) and 3% sucrose were added and stirred until they were dissolved. The 2,4-D (4.42 mg/ℓ) dissolved in ethyl alcohol, and BA (1.13 mg/ℓ) dissolved in 1M NaOH were added. Myoinositol (100 mg/ℓ), thiamine HCl (1 mg/ℓ), nicotinic acid (0.5 mg/ℓ) and Pyridoxine HCl (0.5 mg/ℓ) were added. The pH of the solution was adjusted to 5.8 and heated to boiling without stirring. The agar (0.7%) was added and the solution was heated and stirred until the agar was fully hydrated. The solution was poured into a flask and a sterile filter and dispensing tubes were fixed into the mouth of the flask. The whole was autoclaved for 40 minutes. The glutamine (0.5 g/ℓ) was dissolved in distilled water by stirring and glutamine was sterile filtered into the autoclaved solution. The medium (15 ml) was dispensed into presterilised 2.5 × 10 cm soda glass culture tubes. The tubes were capped with aluminium caps, the medium was allowed to gel and subsequently stored at 2 °C until used.